Low amino acid signaling through the *Saccharyomyces cerevisiae* gene *WHI2* and effects on yeast cell growth

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Abstract

Nutrient sensing is a highly conserved process that evolved for cells to respond to nutrient availability and scarcity. In conditions of low nutrients, cells respond by reducing cellular processes involved in cell growth. The pathways regulating these responses revolve around TOR (target of rapamycin), which is a conserved protein in mammals (mTOR), yeast (Tor1 and Tor2) and *Drosophila* (dTOR). TOR activity decreases in response to low nutrients, but the complex nature of the pathways involved have left many questions unanswered. In particular, how amino acids are sensed to regulate cell growth is incompletely understood.

Our lab identified *WHI2* and the genes for most components of the ESCRT complexes in a genome-wide screen seeking to identify genes required to reduce cell growth in low amino acids. I pursued this thesis project to determine which specific amino acids, if any, were being sensed in a *WHI2*-dependent manner. Several media formulations were modified and new ones were developed to compare the growth of *WHI2* and *ESCRT* knockout strains. Here I provide evidence that leucine is sensed in a ratio to other amino acids to regulate cell growth, and *WHI2* is required for responding to low leucine concentrations. I also show that without *WHI2*, glutamic acid plays a critical role in regulating growth in low leucine concentrations, which could suggest a potential role of *WHI2* in glutamic acid synthesis or metabolism.

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Chapter 1

Introduction to TOR, *WHI2*, and autophagy

Target of Rapamycin

Nutrient sensing is a highly conserved physiological process across essentially all living organisms due to the evolution pressure presented by nutrient scarcity [1]. When amino acid levels in the extracellular environment are low, both mammalian and yeast cells downregulate molecular processes associated with cell growth, such as transcription and translation, while upregulating processes involved in degradation and recycling, such as macroautophagy [1, 2]. The central hub for regulating cell growth in response to nutrient availability is the target of rapamycin (TOR) protein, a large serine/threonine kinase [2–4]. Because several aspects of this pathway are conserved, yeast are a useful model for understanding basic the biology and for delineating potential targets for controlling pathogenic yeast strains.

Saccharomyces cerevisiae have two genes, *TOR1* and *TOR2*, encoding two similar proteins belonging to two different complexes that regulate complementary aspects of cellular growth [4]. Tor1 and Tor2 form the TORC1 (Tor complex 1) and TORC2 (Tor complex 2) [4]. Conversely, mammalian cells only encode one TOR protein, mTOR (mammalian/mechanistic target of rapamycin) [5]. The single TOR protein in mammals forms the two distinct complexes, mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2) [2, 6, 7]. When activated, mTORC1/TORC1 trigger increased transcription, translation, anabolism and lipid biogenesis, and decreased

autophagy, while TORC2/mTORC2 results in increased cell survival, metabolism and actin polymerization and reorganization.[5]

TORC1

While yeast and mammalian TOR complexes have similar functions, the components between the yeast and mammalian complexes are only partially conserved. In mTORC1, mTOR is bound to Tti1/Tel2 (believed to be a scaffolding protein), mLST8 (the mTOR-associated protein, Lethal with *SEC* thirteen, yeast Lst8 homolog) and two kinase inhibitors, DEPTOR (DEP domain containing mTOR-interacting protein) and PRAS40 (a proline-rich Akt substrate of 49 kDa, also called AKT1S1) [2, 7]. mTORC1 also contains RAPTOR (regulatory associated protein of mTOR), while mTORC2 is distinguished by containing RICTOR (rapamycin-insensitive companion of mTOR) (**Fig. 1a**) [2, 3]. Yeast TORC1 consists of Tor1 bound to TCO89, Lst8 and Kog1, the yeast homologue of RAPTOR (**Fig. 1b**) [4].

mTORC1 and TORC1 upstream interactions

As a master regulator of cellular growth, there are many upstream pathways that activate mTORC1/TORC1. However, many of the activating signals converge on the small GTPase Rheb (Ras homolog enriched in brain) [2]. Rheb is found in both mammalian and yeast cells and when activated, acts to activate mTORC1/TORC1, but the mechanism is still unclear [8, 9]. GTP-bound Rheb is believed to upregulate mTORC1 activity by preventing the association of mTORC1 with FKBP38, a protein similar to FKBP12 that binds to mTOR and inhibits mTOR activity [9]. In mammalian

cells, directly upstream of the Rheb GTPase is the negative regulator complex, a dimer of TSC1 (tuberous sclerosis complex 1) and TSC2 [2]. It has been shown that TSC2 acts as a negative regulator of Rheb through its selective GAP activity towards Rheb, which lowers the amount of GTP-bound Rheb [9].

Upstream of the TSC1/2 and Rheb axis is the protein kinase Akt, which is also called PKB (protein kinase B) [4]. Akt is activated through phosphorylation by PDK1 (3-phosphoinositide-dependent protein kinase-1) and PDK2 [4, 9]. However, aside from the direct phosphorylation of Akt by the PDK kinases, the action of TORC2 is also required [4]. TORC2 can phosphorylate the same site as PDK2 on Akt, which promotes phosphorylation by PDK1 [4]. The activation of Akt is believed to be insulin-dependent, and plays an important role in glucose uptake of the cell [11]. It is believed that cell membrane RTKs (receptor tyrosine kinases), GPCRs (G-protein-coupled receptors), and cytokine receptors activate PI3K (phsophoinositide 3-kinase), which will phosphorylate membrane phosphatidylinositol lipids, such as PDK1 and PDK2, that lead to the activation of Akt [11]. The importance of Akt in insulin-signaling and glucose uptake points towards the Rheb/TSC1/2 axis activation of TOR playing a larger role in response to glucose than to amino acids.

Amino Acid Sensing Upstream of mTORC1/TORC1

Recent biochemical dissection of amino acid-dependent activation of TOR has been done best in mammalian systems, but many of these factors were first identified through yeast genetic studies [2]. Leucine has been shown to play an important role in TOR activation [2]. However, other amino acids presumably also play a crucial role, as

cells do not activate TOR in the presence of only leucine [2]. However, the other amino acids that play a role with leucine to regulate growth are still unknown, although glutamine cotransport with leucine has been reported in mammalian cells [2]. Research from several groups suggest that the lysosome (yeast vacuole), the primary subcellular reservoir of amino acids, is the site where amino acids are sensed to regulate TOR activity and growth in mammalian and yeast cells [11–16]. Supporting these claims, mTORC1/TORC1 is commonly localized to these organelles [2, 17].

The proteins that control mTORC1 localization to the lysosome are the GTPase heterodimers Rag A/B and Rag C/D [2]. When the cell is deprived of amino acids, RagA/B binds GDP, which is exchanged for GTP when the cell is no longer starved of amino acids [2]. This binding of GTP recruits both the Rag heterodimers and mTORC1 to the lysosomal membrane [2]. Although yeast TORC1 is constitutively localized to the vacuole membrane, the Rag orthologs in yeast, Gtr1 (GTP binding related 1) and Gtr2, also activate TORC1 when amino acids are abundant [2, 3].

While the Rag proteins and the Gtr proteins are important for activating mTORC1, these proteins do not contain lipid localization domains to guide them to the lysosomal or vacuolar membrane [2]. The localization process is controlled in mammalian cells by a complex called the Ragulator, with the functional analog in budding yeast, *S. cerevisiae*, suggested to be the heterodimeric complex called EGO1/3 [2, 18]. The Ragulator complex preferentially binds to the inactivated, unbound or GDP-bound RagA/B complex, and consequently loads a GTP into the complex (**Fig. 2**), activating the Rag heterodimers [2]. In yeast, it is believed that the EGO complex plays a related function as the Ragulator, although it does not have sequence similarity [3]. The

structural similarity of Ego3 to components of the Ragulator complex, as well as the lipidation of Ego1 suggest that it also plays a role in tethering the Gtr proteins to the vacuole [3, 18]. However, a separate GEF and not the EGO complex has been proposed to activate Gtr1 [3].

These factors function proximally in the TOR pathway to respond to amino acids levels, but less is known about the upstream pathway. It was discovered, through screening dTORC1 interactors, that V-ATPase is required to control Ragulator function [2]. However, how it regulates the Ragulator complex is still unknown, as its established role in acidifying lysosomes does not appear to be important in amino acid regulation of mTORC1 [2]. In yeast, V-ATPase not only plays a similar function in acidifying the vacuole, it has also been shown to play an important role in vacuole permeabilization in cell death [20]. While this permeabilization occurs in yeast cellular death, V-ATPase may play a similar role in TOR activation by allowing certain amino acids to move through a permeabilized vacuolar membrane and activating upstream TOR pathways.

Under low nutrient conditions, the GATOR1 complex inactivates RagA/B by stimulating hydrolysis of bound GTP, as inhibition of GATOR1 caused cells to have constitutively active mTORC1 even in low amino acid conditions [2, 20]. The GATOR1 complex consists of DEPDC5, Nprl2, Nprl3, which is inhibited by the GATOR2 complex, comprised of Mios, WDR2, WDR59, Seh1L, Sec13, and most of these have yeast orthologs [2, 20]. Yeast Npr2/3 were previously identified in a yeast screen for genes required to respond to nitrogen starvation, and were also identified in our lab's screen (**Fig. 2**) [2, 20].

The Sabatini group also recently discovered a putative arginine transporter, called SLC38A9.1, on the lysosomal membrane that regulates the process of GTR activation by GATOR1 (**Fig. 2**) [14]. SLC38A9.1 was shown to have arginine transport capability, and the non-membrane associated end seems to preferentially bind to GDP-bound RagA/B, similarly to the Ragulator [2, 13]. When considered together, the hypothesis made by the Sabatini group is that the binding of arginine, and possibly leucine, could lead to a conformational change in the membrane-associated domain [14]. This change in conformation could be relayed to the Ragulator to activate the Rag heterodimers, but the mechanisms behind this are still unknown [14]. Even with the discovery of this gene, there is still incomplete information about how other amino acids are sensed to activate TORC1 and how the activation occurs.

mTORC1/TORC1 downstream effectors

Although it is unclear how nutrient conditions are sensed by mTORC1/TORC1, the downstream effectors of TOR activation leading to cellular growth are also actively studied. The activation of mTORC1/TORC1 leads to the direct phosphorylation of several targets, including Sch9 and Tap42 [4, 8, 21–23]. The phosphorylation of these proteins leads to the cellular processes involved in growth [4, 8, 21–23].

Sch9 in yeast, and S6 kinase (S6K) in mammalian cells, is the primary kinase that bridges TORC1 activation and protein translation [4, 8, 21–23]. In mammalian cells, activation of S6K has been implicated in promoting of increased of 5'-terminal oligopyrimidine tracts (5'TOP) mRNA translation, which exclusively encodes for proteins of the translation machinery [8]. S6K also indirectly increases mRNA translation

through phosphorylation of downstream effectors, such as the conserved ribosomal protein S6 [8]. In yeast, upon TORC1 activation, Sch9 becomes activated through direct phosphorylation by TOR, which goes on to phosphorylate the ribosomal protein S6 [4, 23]. While presumed to have a role in translation, the role of phosphorylated S6 is still debated as some studies have shown that other kinases can phosphorylate S6 in a TOR knockout system [8]. Another target of activated S6K/Sch9 is the phosphorylation of eIF4B, which goes on to promote cell growth and proliferation (**Fig. 3**) [8].

In yeast and mammalian cells, Tap42 acts in a complex with PP2A (protein phosphatase 2a) directly downstream of TORC1 to regulate a wide variety of cellular functions [22, 23]. The Tap42-PP2A complex regulates amino acid synthesis and nitrogen through proteins such as Npr1, Gln3 (Glutamine metabolism 3), Rtg1/Rtg3 and other factors (**Fig. 3**) [23]. Additionally, the complex also regulates cellular responses to environmental stress through the Msn2/Msn4 pathway (**Fig. 3**) [23]. Tap42 has been shown to repress these pathways when activated TORC1 directly phosphorylates Tap42 to stimulate binding to PP2A, consequently inactivating PP2A's role in the amino acid synthesis and autophagy pathways (**Fig. 3**) [24].

Tap42 has also been implicated in regulating the autophagy pathway through the Atg13-Atg1 complex, but there is conflicting data suggesting that Tap42 may not be necessary in autophagy induction upon TORC1 inactivation [23–25]. This link between autophagy and TOR is of particular interest to this project as Whi2, a yeast gene, has been shown to play a role in autophagy function [27]. Autophagy is a process the cell undergoes to degrade micronutrients or damaged cellular components for reuse in other anabolic processes [28]. According to Ohsumi, the group observed that TORC1 directly

phosphorylated Atg13, preventing it from forming the Atg1-Atg13 complex required to induce autophagy [25]. On the other hand, the Klionsky group showed that upon inactivation of PP2A via Tap42, Atg1 is indirectly inhibited through an unknown mechanism [26]. These findings thus make the role of Tap42 in autophagy regulation unclear.

The pathways controlled by TORC1 activation lead to increased translation and transcription through Sch9 and Tap42 activation, and decreased autophagy through phosphorylation of the Atg1-Atg13 complex. The regulation of these pathways is particularly important so that the catabolic processes upregulated by TORC1 are not immediately degraded by the autophagic process, and conversely, when the cell is not actively synthesizing new molecules, the autophagy process can provide recycled nutrients needed to sustain cellular functions [6].

Interestingly, a recent study has shown that the downstream effectors of TORC1 activation are partially dependent on the nutrient source that is regulating TORC1 [23]. While rapamycin exposure or nitrogen starvation did not downregulate the PP2A pathway, glucose starvation conditions resulted in only weakly activated PP2A pathways [23]. However, regardless of starvation and stress conditions, the Sch9 pathway was inhibited [23]. This effect was regulated by the upstream activators of TORC1, Gtr1/2, Npr2/3, and Rho1 (a protein involved in TORC1 and PP2A association), which are involved in TOR1 activation in a nitrogen/amino acid-dependent manner. Snf1 plays a critical role in shutting off TORC1 in glucose-deprived environments, while Hog1 was identified as a stress-activated protein that inactivates TORC1 in response to osmotic stress, but not other stress or starvation conditions [23]. This is particularly interesting as

it ties the growth phenotype to specific types of starvation or stress encountered by the cell. While this ties the upstream effectors of TORC1 to the downstream response pathways, the genes involved in the TORC1 response to stress and starvation have yet to be identified.

WHI2 in cell growth and autophagy

WHI2 was reported to be a fungal-specific gene discovered in the late 1980's [29]. Little is known about *WHI2* except that it plays roles in regulation of cell morphology, stress responses and control of cell growth [26, 29–32]. One reason that *WHI2* is poorly studied is because it was believed to lack homologues or orthologs in humans. However, our lab reported that Whi2 is homologous (predicted to have a common ancestor) to a family of 25 human KCTD (potassium channel tetramerization domain) proteins, due to similarities in the BTB domain (**Fig 4**) [34].

Many of the known downstream effectors of Whi2 are involved in the stress response and cell growth [28–30]. Whi2 is reported to be involved with the phosphorylation of the stress response-related transcription factor Msn2, as well as the plasma membrane-associated phosphatase Psr1 [34, 35]. A study indicated that in cells deleted for *WHI2*, Msn2 was found to be hyperphosphorylated [36]. Both Psr1 and Msn2 are necessary for activating the stress response.

Most of the early literature about *WHI2* was from the Sudbury group, which showed that cells with *whi2* mutations tended to grow faster, and were smaller in size, than wild-type cells when grown in ethanol [37]. Additionally, in carbon starvation conditions, the *whi2* mutant cells did not arrest cellular growth like the wild type cells due to dysregulation of the G1 cyclins, CLN1 and CLN2 [37]. The Sudbury group, using glucosamid and glucose to tease apart the regulation of growth due to low glucose or catabolite repression in *whi2* mutants, came to the conclusion that Whi2 plays a role as a negative regulator of cell growth [31]. Lastly, their group showed that overexpression of *WHI2* not only resulted in cell cycle arrest, but also resulted in a filamentous

morphology, which may be the result of improper budding [30, 31]. A recent study on Pus1, a protein involved in yeast gene splicing, reported that *PUS1* overexpression also resulted in filamentous growth [35]. The presence of a STRE (stress response element) promoter region upstream of PUS1, seems to indicate that growth and division may be regulated by Whi2 and its interacting partner Psr1 in the stress-response pathway [35].

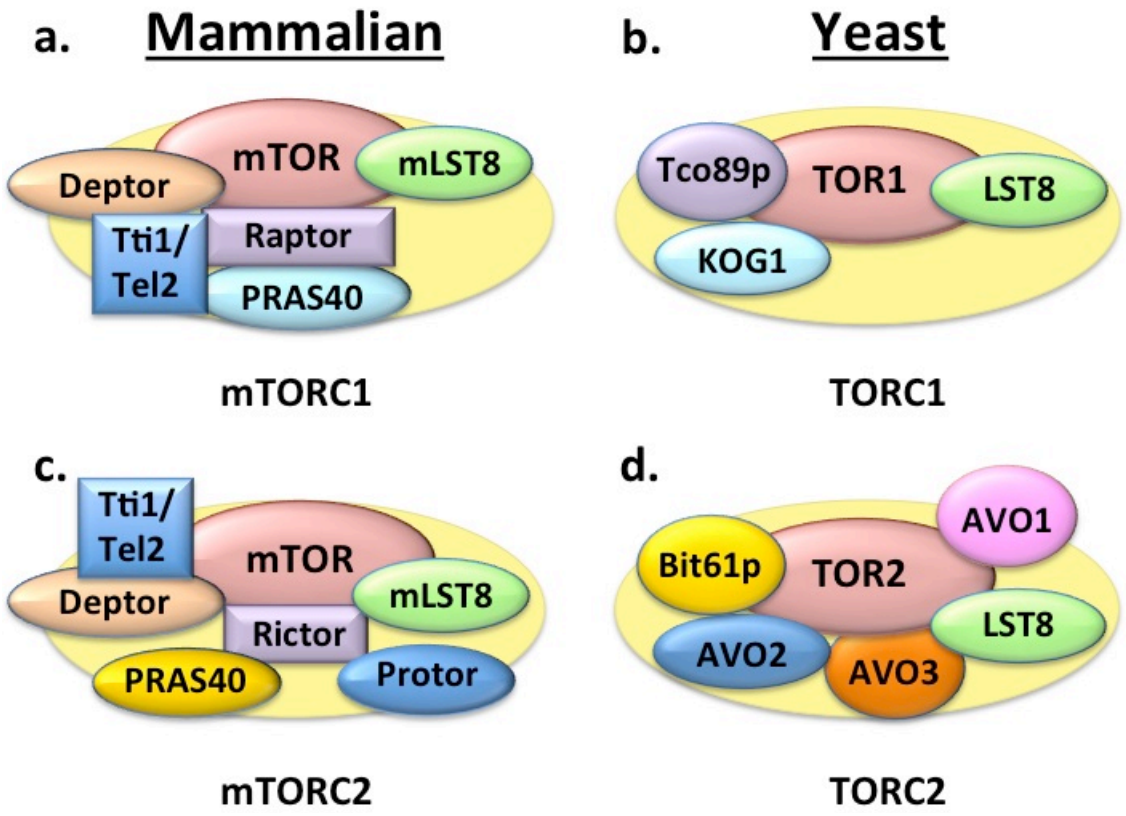
Two recent studies on *WHI2* disagree on its role in autophagy, in particular its role in mitophagy. Autophagy is classified into three main types, macroautophagy, microautophagy, and mitophagy, which is a similar process targets mitochondria for degradation via the autophagosome [38]. Macroautophagy focuses on recycling and degrading damaged organelles, cytosolic proteins and invasive microbes, through capture by a specialized vesicle called the autophagosome [39]. Conversely, microautophagy directly uptakes degradation targets by the vacuole or lysosome, and typically targets soluble components, but can help degrade damaged organelles [39, 40].

The link between Whi2 and mitophagy was suggested by the Reichert group, who reported a mitophagy defect in the *Dfis1* deletion strain. They attributed the mitophagy defect to the secondary mutation in *whi2* that was previously reported by our lab to occur in *Dfis1 strains* [26, 32]. Reichert and colleagues showed that mitophagy, and to a certain extent autophagy, was not as robustly induced in a Δ*whi2* strain when Tor1 was inhibited by rapamycin [27]. The decrease in mitophagy was rescued in both the Δ*fis1* and Δ*whi2* strains with the complementation of *WHI2*, but not *FIS1* [27]. A mitophagy defect is also suggested by the electron microscopy images our lab reported for the *Dfis1* deletion strain [42]. This also seems to be confirmed with a higher prevalence of mitochondria with defective membrane potentials in Δ*whi2* cells compared to wild type cells [27]. This

is consistent with our lab's earlier report that a mutation in *whi2* rescues mitochondrial phenotypes of Δ*fis1* [33]. However, a role for *WHI2* in mitophagy or autophagy is controversial. Klionsky and colleagues reported that deletion of *whi2* did not affect mitophagy, but instead required the fission function of *FIS1* when autophagy was induced by a classical stimulus nitrogen starvation [42, 43].

Our lab performed a genome-wide screen to identify knockout strains that grow significantly better than wild type on low amino acid medium. The top hit in this screen was the knockout of *WHI2,* and this screen also identified TORC1 negative regulators *NPR2/3* mentioned above. This same screen for genes required to reduce cell growth in low amino acids identified almost all of the components of the ESCRT pathway [34]. The ESCRT complexes are a series of 5 complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and the vps4 complex (ESCRT-IV), that are important for endosomal trafficking, vesicle sorting and other functions [45]. In yeast, they play an integral role in delivering cargo to the vacuole, which is also the primary organelle where nutrients are stored and cellular components begin degradation [45]. Cierra Sing, a previous masters student in our lab, tested and found that a deficiency in ESCRTs leads to a defect in nutrient sensing Knockouts of ESCRT complex components results in an overgrowth phenotype similar to what is observed of the Δ*whi2* [46].

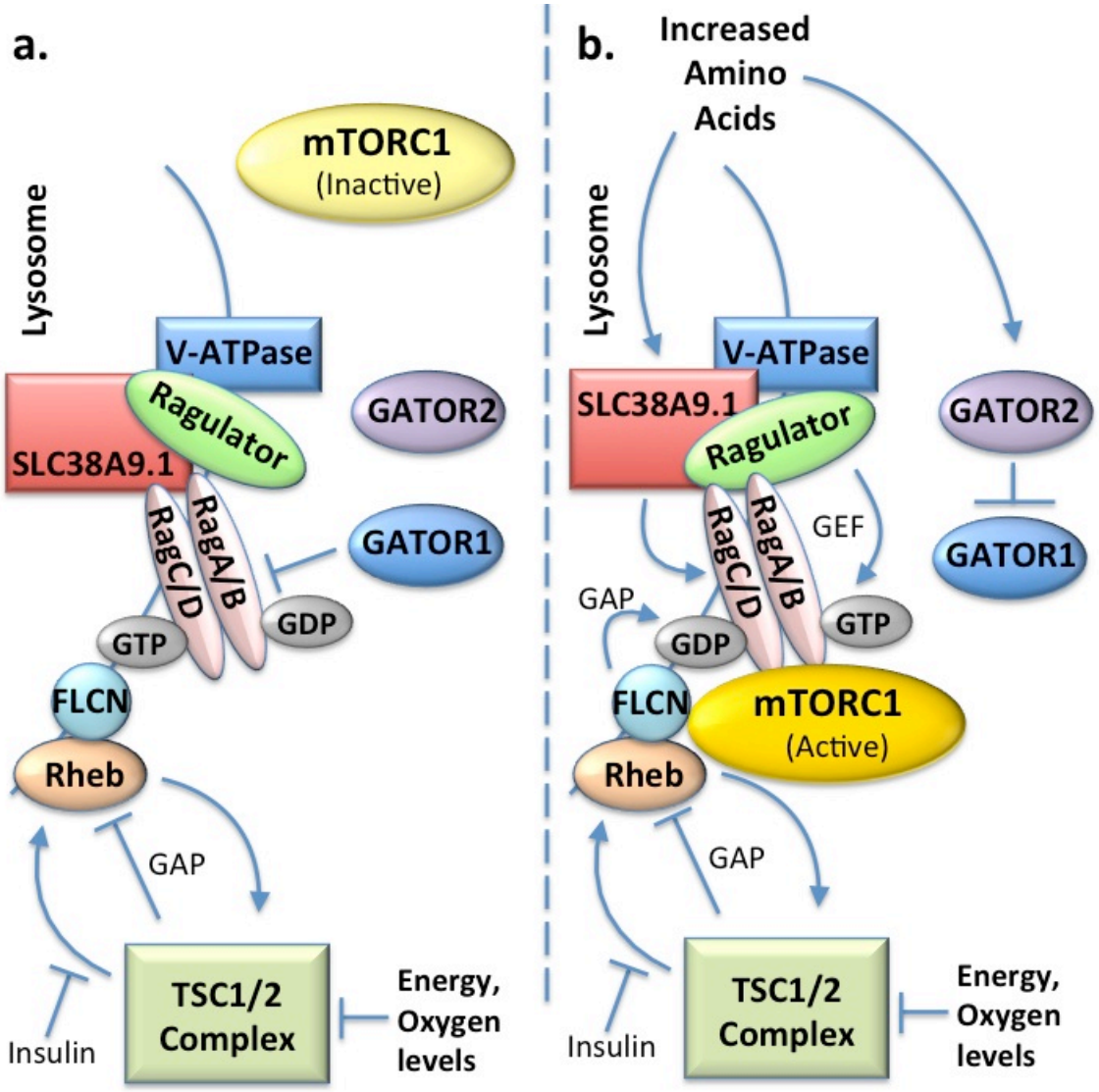
Figure 1



Adapted from Bar-Peled et al. and Cuyàs et al.[2, 7].

Figure 1 – TOR complexes in mammalian and yeast cells
a) mTORC1 in mammalian cell. mTOR is bound with mLST8, DEPTOR, RAPTOR, PRAS40, and Tti1/Tel2. **b)** TORC1 in yeast cell. Tor1 is bound to Lst8, Tco89p, and Kog1. **c)** mTORC2 in mammalian cell. mTOR is bound with Tti1/Tel2, mLST8, DEPTOR, and PRAS40 (similar to mTORC1). It is also bound to RICTOR and PROTOR. **d)** TORC2 in yeast cell. Tor2 is bound to Lst8 like TORC1. Tor2 is also bound to Avo1, Avo2, Avo3, and Bit61. Figure Adapted from Bar-Peled et al. and Cuyàs et al. [2, 7].

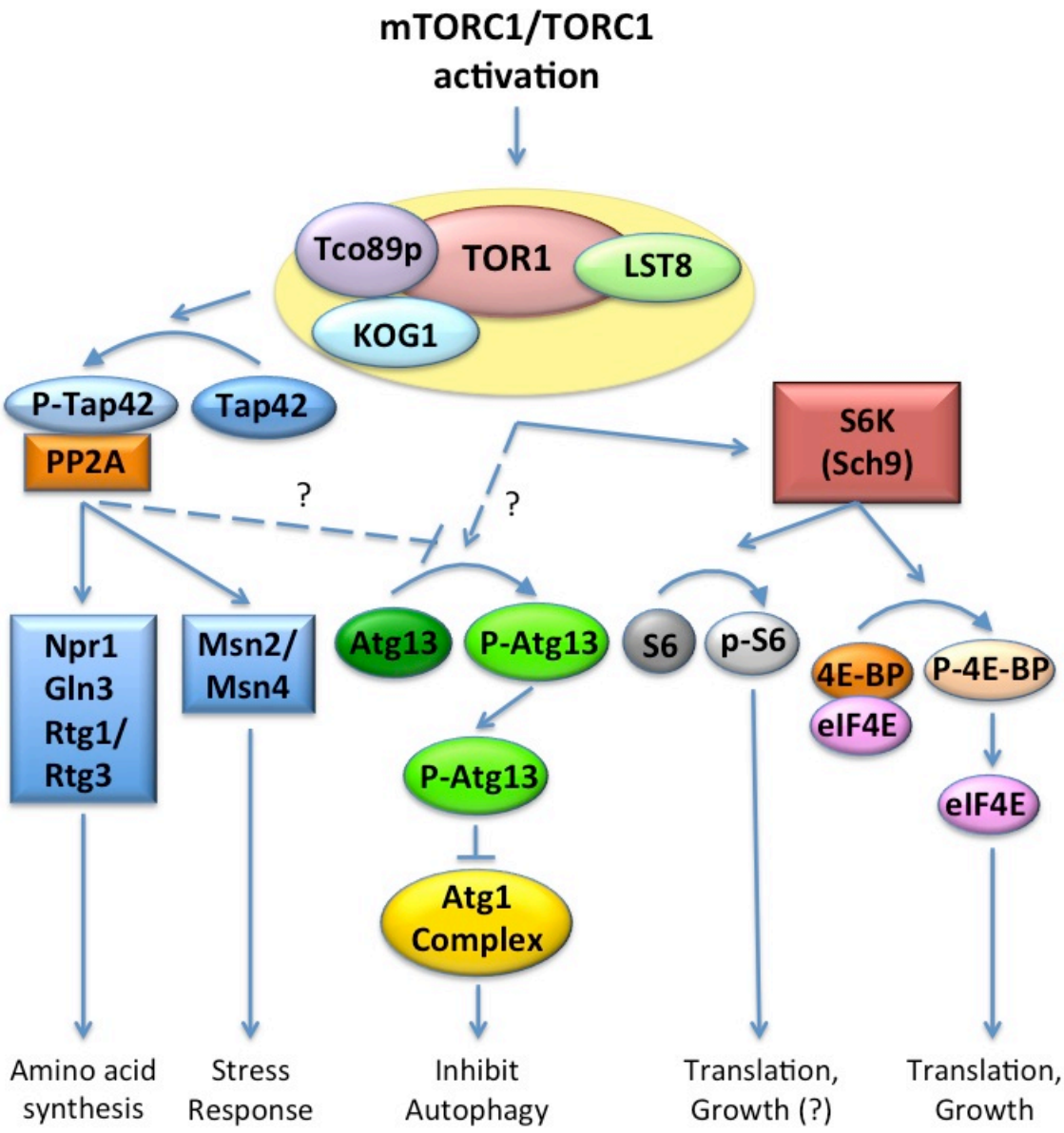
Figure 2



Adapted from Bar-Peled et al. and Brown et al.[2, 13].

Figure 2 – Upstream pathway in amino acid dependent activation of mTORC1
a) mTORC1 inactivation in low amino acid conditions. RagA/B is GDP bound, and the GEF activity of the Ragulator is inhibited by GATOR1, and mTORC1 is not localized to the lysosomal membrane. The TSC1/2 complex is still interacting with Rheb, regardless of amino acid conditions. SLC38A9.1 is bound to the Ragulator, but not activated. **b**) mTORC1 activation in response to amino acids. GATOR2 inhibits GATOR1, allowing the Ragulator to exchange GDP on RagA/B for GTP. GTP-bound RagA/B recruits mTORC1 to the lysosomal service, which can be activated by the TSC1/2-Rheb pathway. Additionally, high arginine may cause conformational changes SLC38A9.1, which is relayed to the Ragulator. Or, the non-membrane associate end may associate with RagC/D to localize TOR. The exact function of SLC38A9.1 is still unclear. Figure adapted from Bar-Peled et al. and Brown et al. [2, 13].

Figure 3



Adapted from Hughes Hallet et al.[23].

Figure 3 – TORC1/mTORC1 downstream pathways

Upon TORC1/mTORC1 activation different proteins are phosphorylated resulting in the changes to cellular processes that increase growth. TORC1 directly phosphorylates Sch9 and Tap42, which in turn regulate the multiple downstream proteins to regulate amino acid synthesis, stress response, autophagy, translation, transcription, and growth. Figure adapted from Hughes Hallet et al. [23].

Figure 4

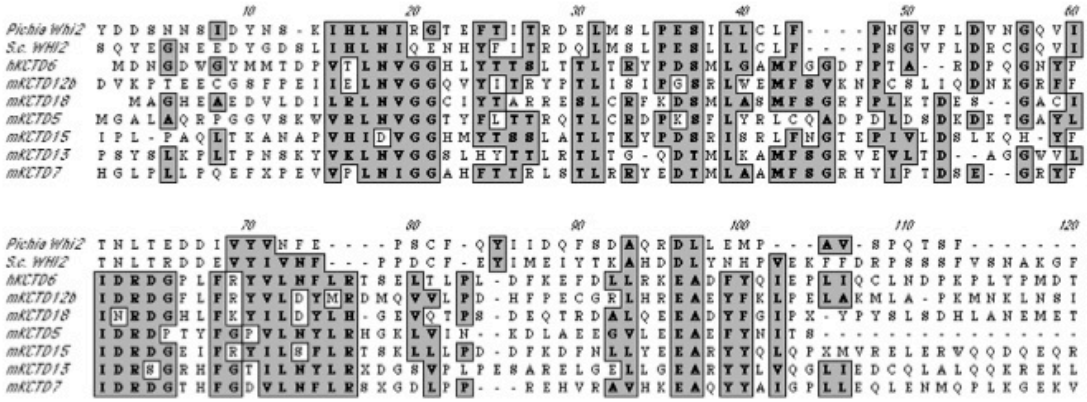


Figure 4 – Alignment of N-terminal end of yeast Whi2 and human KCTDs
Protein sequence alignment of the N-terminal end of Whi2 from two yeast strains, *S. cerevisiae* and *S. pombe*, with various human KCTD proteins. The N-terminal end of the KCTDs contains the BTB (BR-C, ttk and bab) domain, which is a binding domain highly conserved across species.

Chapter 2

Materials and Methods

Yeast Strains and Media

All knockout yeast strains used have a BY4741 background with engineered auxotrophies (**Table 1**). The background parental "wild type" strains, unlike wild strains, which are prototrophs for all 20 amino acids, were engineered to be auxotrophs for four amino acids (histidine, lysine, leucine and methionine) and uracil. Similarly, human cells are auxotrophic for phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine [46, 47].

The rich medium YPD [1% yeast extract (Fisher Scientific), 2% peptone (Peptone-Y, MP Biomedicals), 2% D-glucose/dextrose (Fisher Scientific)] was used to grow all un-transformed strains in preparation for experiments. All SCD media, including low amino acid media and amino acid dropout media, contained 0.67% yeast nitrogen base without amino acids (BD and Sigma-Aldrich), 0.2% glucose (Fisher Scientific), and different combinations of dissolved amino acid solutions (**Table 2 and Appendix 1).** Two sets of media were made independently from dissolved amino acids to confirm the results. Amino acids were dissolved in liquid at 1.0 g/100 mL (except cysteine at 0.1 g/10 mL, aspartic acid at 0.5 g/100 mL, and tyrosine at 0.1 g/100 mL) and sterilized by filtration with 0.2μm filter. To reduce potential errors, dropout mixes were commonly shared between recipes where possible. YPD and SCD (synthetic complete dextrose) agar plates were made by adding 2% agar (BD) to liquid YPD. All media was steamed autoclaved at 121^{o}C for 15 minutes. The dropout selection occurs via the omission of

auxotrophic nutrients from the media, selecting against cells that cannot synthesize the missing nutrient. There are benefits over drug or other selection and have been well documented, such as the lack of protein expression that may cause protein aggregation [49].

Yeast Serial Dilution Plating

All wild type and knockout yeast strains (**Table 1**) were streaked from frozen 15% glycerol stocks without thawing onto YPD agar plates and allowed to incubate at 30°C for 2 days. Transformed strains were streaked from frozen 15% glycerol stocks without thawing onto SCD_{CSH}-URA agar plates and allowed to incubate at 30°C for 2 days. Single colonies were picked from the plates and inoculated into 3 mL of YPD (SCD_{CSH}-URA for transformed cells) and grown overnight (at least 14 hours) at 30°C on a rotator. OD_{600} for each yeast culture was then measured using Thermo Scientific® Genesys 10s Vis Spectrophotometer. Overnight cultures were initially diluted to 0.5 OD_{600}/mL with water to ~100uL total volume, then serially diluted 1:5 5 times with water, and 3 μL of the original 0.5 OD_{600} dilution and 5 serial dilutions were plated onto SCD_{CSH}, SCD_{ME}, and other plates with the indicated amino acid compositions. The plates were incubated at 30°C and images were taken and archived after 3 days of incubation. Images shown in the figures presented were 0.05 second exposures taken using a Bio-Rad® Universal Hood III imaging system and the ImageLab 5.0 software after the 3 day incubation. All plates shown are representative images from experiments that have been repeated at least 2 times for an $n \geq 3$, unless indicated otherwise. Contrast and brightness

were both increased by 20% each in PowerPoint to increase visibility, unless otherwise noted.

Plasmid Transformation

Wild type and Δ*whi2* strains were transformed with BQ23 (empty vector with *URA3* selection cassette), TXC19 (BQ23 vector expressing HA-Whi2), or GFP-Atg8 plasmids using the following established lab protocol. An overnight 3 mL yeast culture in YPD (grown at least 14 hours at 30°C on a rotator) was diluted to prepare 2 mL yeast cultures of 0.20 OD_{600}/mL in YPD, which were incubated at 30°C for 3-4 hours or until the OD_{600} was between 0.5-1.0 OD/mL. OD_{600} for each yeast culture was then measured using Thermo Scientific® Genesys 10s Vis Spectrophotometer. 1 mL of yeast culture was centrifuged using a Sorvall® Biofuge *Pico* model microcentrifuge at 13,000 RPM for 2 minutes; the pellet was washed once with 0.1 M LiAc, and resuspended with 100 μL of LiAc. 5 μL per transformation of denatured salmon sperm DNA (ssDNA) was prepared by boiling at 100°C for 5 minutes and place on ice. 1-2 μg of plasmid DNA, 5 μL ssDNA and 350 μL of transformation plate mix were added to the resuspended yeast cells. The mixture was incubated for 30 minutes 30°C on a rotator. After the incubation, the sample was heat shocked for 10 minutes in a 42°C water bath. The samples were centrifuged at 13,000 RPM for 2 minutes, resuspended in 100 μL of water, and spread onto a selective yeast plate (SCD_{CSH}-URA, Appendix 1). Plates were incubated for 2 days at 30°C.

Table 1 Yeast strains used in this study

Strain Name	Background	Genotype	Notes
WTα	BY4742	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	Glycerol stock obtained from Jef Boeke lab
WTa	BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Glycerol stock obtained from Jef Boeke lab
Δwhi2 ss02_05_	BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ1*	Substrain stock made from clean KO verified by tetrad analysis done previously by Xinchen Teng.
Δvps4 ss03_18	BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ2*	Substrain stock made from clean KO verified by tetrad analysis done previously by Cierra Sing.
Δvps27 ss15_08	BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ3*	Substrain stock made from clean KO verified by tetrad analysis done previously by Cierra Sing.
Δvps28 ss08_11	BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ4*	Substrain stock made from clean KO verified by tetrad analysis done previously by Cierra Sing.
Δsnf7 ss01_06	BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ5*	Substrain stock made from clean KO verified by tetrad analysis done previously by Cierra Sing.
Δsnf8 ss08_14	BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ6*	Substrain stock made from clean KO verified by tetrad analysis done previously by Cierra Sing.

Table 2 Basic SCD Recipes

	SCD_{CSH}			SCD_{ME}			SCD_{Dawes}		
	mg/L	Analysis		mg/L	Analysis		mg/L	Analysis	
		Leu % of Total AA	Specific AA to Leu ratio		Leu % of Total AA	Specific AA to Leu ratio		Leu % of Total AA	Specific AA to Leu ratio
Adenine	18.35			20			10		
L-alanine	73.4	4.17	0.2	0	0	0	0	0	0
L-arginine	73.4	4.17	0.2	20	1.67	0.67	50	6.17	0.5
L-asparagine	73.4	4.17	0.2	0	0	0	0	0	0
L-aspartic acid	73.4	4.17	0.2	100	8.33	3.33	80	9.88	0.8
L-cysteine	73.4	4.17	0.2	0	0	0	0	0	0
L-glutamine	73.4	4.17	0.2	0	0	0	0	0	0
L-glutamic acid	73.4	4.17	0.2	100	8.33	3.33	0	0	0
L-glycine	73.4	4.17	0.2	0	0	0	0	0	0
L-histidine	73.4	4.17	0.2	20	1.67	0.67	20	2.47	0.2
Inositol	73.4			0	0		0	0	
L-isoleucine	73.4	4.17	0.2	30	2.5	1	50	6.17	0.5
L-leucine	367	20.83	1	30	2.5	1	100	12.35	1
L-lysine	73.4	4.17	0.2	30	2.5	1	50	6.17	0.5
L-methionine	73.4	4.17	0.2	20	1.67	0.67	20	2.47	0.2
PABA	73.4			0	0		0	0	
L-phenylalanine	73.4	4.17	0.2	50	4.17	1.67	50	6.17	0.5
L-proline	73.4	4.17	0.2	0	0	0	0	0	0
L-serine	73.4	4.17	0.2	400	33.33	13.33	0	0	0
L-threonine	73.4	4.17	0.2	200	16.67	6.67	100	12.35	1
L-tryptophan	73.4	4.17	0.2	20	1.67	0.67	100	12.35	1
L-tyrosine	73.4	4.17	0.2	30	2.5	1	50	6.17	0.5
Uracil	73.4			20	1.67		20	2.47	
L-valine	73.4	4.17	0.2	150	12.5	5	140	17.28	1.4
Total	1761.6			1200			810		

Table 2 Basic SCD recipes (cont.)

	SCD_{MEDD}			SCD_{Aux}		
	mg/L	Analysis		mg/L	Analysis	
		Leu% of Total AA	Specific AA to Leu ratio		Leu% of Total AA	Specific AA to Leu ratio
Adenine	10			0		
L-alanine	0	0	0	0	0	0
L-arginine	20	3.51	0.67	0	0	0
L-asparagine	0	0	0	0	0	0
L-aspartic acid	80	14.04	2.67	0	0	0
L-cysteine	0	0	0	0	0	0
L-glutamine	0	0	0	0	0	0
L-glutamic acid	0	0	0	0	0	0
L-glycine	0	0	0	0	0	0
L-histidine	20	3.51	0.67	20	22.22	0.67
Inositol	0	0		0	0	
L-isoleucine	30	5.26	1	0	0	0
L-leucine	30	5.26	1	30	33.33	1
L-lysine	30	5.26	1	20	22.22	0.67
L-methionine	20	3.51	0.67	20	22.22	0.67
PABA	0	0		0	0	
L-phenylalanine	50	8.77	1.67	0	0	0
L-proline	0	0	0	0	0	0
L-serine	0	0	0	0	0	0
L-threonine	100	17.54	3.33	0	0	0
L-tryptophan	20	3.51	0.67	0	0	0
L-tyrosine	30	5.26	1	0	0	0
Uracil	20	3.51		20	22.22	
L-valine	140	24.56	4.67	0	0	0
Total	570			90		

Figure 5

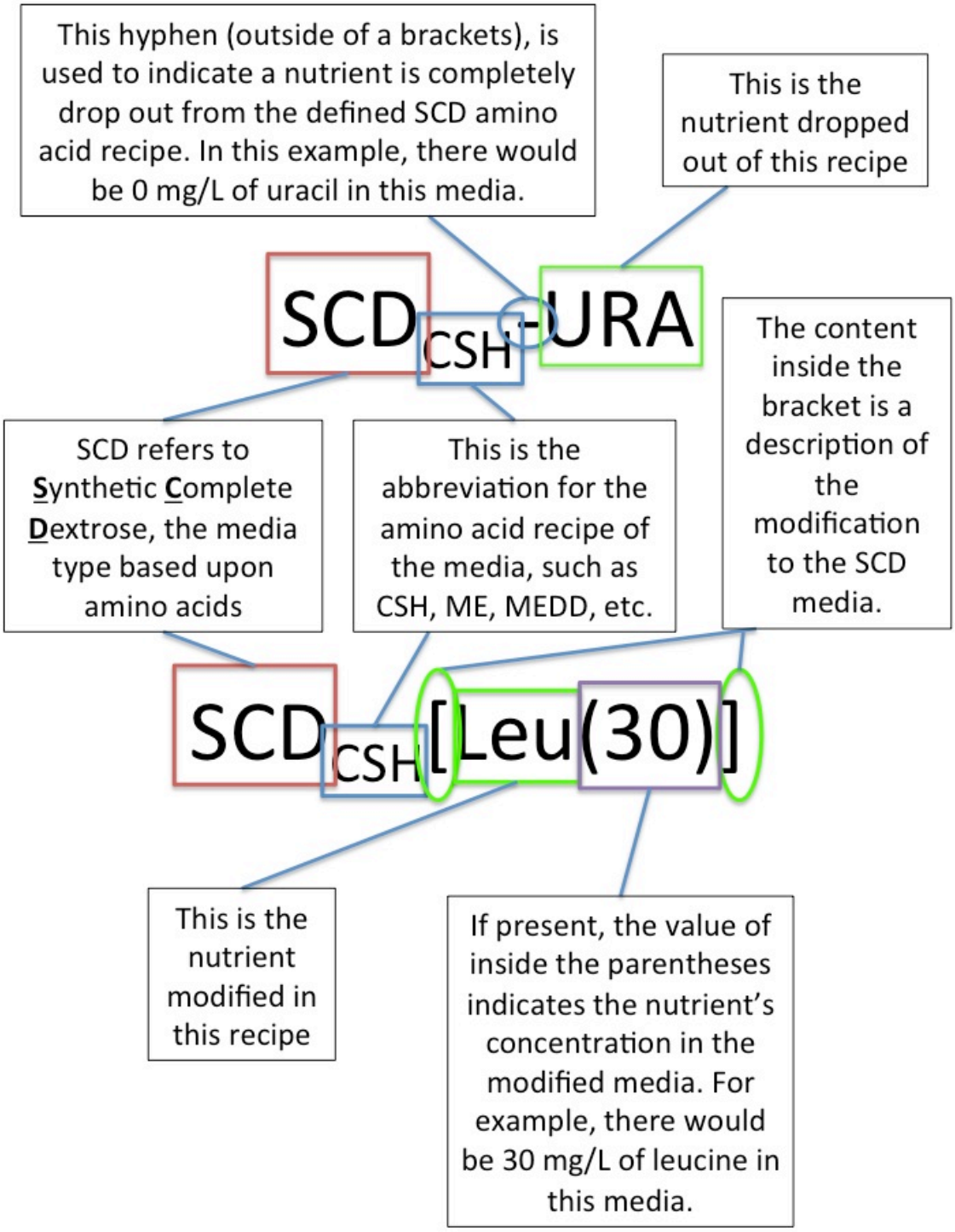


Figure 5 – Defining plate nomenclature used in experiments

Chapter 3

Leucine is sensed in a ratio with other amino acids to regulate cell growth

Introduction

Our lab performed a cell death screen of yeast knockout strains to identify cell death-sensitive/-resistant strains following cell stress. This screen identified Δ*whi2* as a particularly death-sensitive knockout strain, although both Δ*whi2* and wild type strains grew indistinguishably prior to a cell death stimulus (on YPD) [33]. Unexpectedly, the death-prone Δ*whi2* strains grew up to 100-fold better than wild type when plated (without treatment) on a low amino acid synthetic complete medium with dextrose (SCD) prepared by the recipe published in Methods in Enzymology, therefore designated SCD_{ME} (**Table 2**) [50]. This recipe has lower total amino acid levels than the SCD recipe published by Cold Spring Harbor Press SCD_{CSH}, where both strains grow indistinguishably (**Table 2**) [33]. This suggested that Δ*whi2* mutants fail to sense or respond to low amino acids [34]. Therefore, a genome-wide screen of the knockout collection (BY4741 background) was performed to identify all the genes in yeast required to slow cell growth in low amino acid medium. In this screen, 751 unique knockout strains were found to substantially overgrow compared to wild type [34]. This failure to arrest growth under low amino acid conditions, as exhibited in Δ*whi2* mutant cells, is referred to here as an "overgrowth" phenotype.

Given that amino acids were known to be a potent activator of mTORC1 and TORC1, Wen-Chih Cheng in our lab explored possible involvement of TORC1 [34]. Margaret Dayhoff-Brannigan and others in the lab showed that supplementing the SCD_{ME} media with leucine to 300 mg/L, thereby mimicking the high leucine concentration in SCD_{CSH} (367 mg/L, **Table 2**), could rescue WT growth to match that of Δ*whi2* mutants [51]. Therefore, leucine was sufficient to restore otherwise restricted growth of wild type strains. they also performed the inverse experiment, reducing the leucine in SCD_{CSH} to match low levels in SCD_{ME}, 30 mg/L. As predicted, Δ*whi2* mutants grew better than wild type, but curiously, the overall growth for all strains was severely reduced despite having higher total amino acid levels [51]. I found this intriguing, as it seemed to indicate that the cell growth responded to amino acids other than L-leucine. Therefore, I investigated this and other conditions to characterize the amino acid-sensing function of *WHI2 and ESCRTs*.

Results

WHI2 and ESCRT complexes are required to respond to low leucine

Low amino acid medium (SCD_{ME}) causes wild type yeast to slow their growth [33]. The slow growth is not due to insufficient nutrients because our lab found that deletion of *WHI2* results in a dramatic overgrowth phenotype. I recapitulated these findings, showing that *Δwhi2* mutants grow far better than wild type on SCD_{ME} (**Fig. 6b**) but not SCD_{CSH}, where wild type and *Δwhi2* grow indistinguishably (**Fig. 6a**). The two greatest differences between the two media SCD_{ME} and SCD_{CSH} is the total amino acid concentration (1200 and 1761 mg/L, respectively), and the most obvious difference

among individual amino acids is the leucine concentration, which is ~10-fold lower in SCD_{ME} compared to SCD_{CSH} (30 mg/L vs. 367 mg/L, respectively) (**Table 2**). Given the importance of leucine sensing in mammalian cells, we suspected that *WHI2* might be specifically required to respond to low leucine concentrations. However, a 10-fold difference in any other amino acid could potentially have the same effect, as the experiments performed thus far did not definitively distinguish between these possible explanations. Therefore, I designed and tested a number of different media that differ in amino acid content and composition to determine which amino acid(s) specifically require yeast *WHI2* to appropriately reduce cell growth in the face of nutrient deprivation. I analyzed the growth of WT, Δ*whi2*, and representative knockout strains from different ESCRT complexes to achieve this goal, revealing some additional surprising findings. The knockout strains used in this project lack Vps27 (found in ESCRT-0), Vps28 (found in ESCRT-1), Snf8 (found in yeast ESCRT-II), Snf7 (found in ESCRT-III), or Vps4 (found in ESCRT-IV) [45]. Note that tetrad analyses were performed on all of these strains to verify that the knockout gene itself is responsible for the overgrowth phenotype [46].

Because the leucine concentration of SCD_{CSH} is exceptionally high compared to other amino acids in SCD_{CSH} and when compared to leucine levels in SCD_{ME}, and because leucine is a known regulator of cell growth we considered that yeast *WHI2* is required to slow cell growth specifically in low leucine [2]. To investigate this possibility, I prepared plates in which all components remained the same except that the leucine concentrations were reversed between SCD_{ME} and SCD_{CSH}, similar to those in the lab before me. The leucine concentration of SCD_{CSH} plates (367 mg/L Leu) was lowered

to reflect the amount of leucine in the SCD_{ME} plate (30 mg/L Leu), and the concentration of leucine in the SCD_{ME} plate (30 mg/L) was increased to 367 mg/L. Lowering the leucine concentration of SCD_{CSH} revealed an overgrowth phenotype by Δ*whi*2 and the ESCRT knockout strains, although the growth of all strains was unexpected reduced (**Fig. 6c**). Conversely, increasing the amount of leucine in the SCD_{ME} recipe to 367 mg/L abolished the overgrowth phenotype typical of SCD_{ME}, and all strains grew similarly and robustly (**Fig. 6d**). These results confirmed previous findings in our lab and implicate leucine as a key influence on the Δ*whi2* overgrowth phenotype [51].

While inverting the leucine concentrations in SCD_{CSH} or SCD_{ME} was sufficient to affect the overgrowth phenotype of the Δ*whi*2 and ESCRT knockout strains, changes made to leucine concentrations also changed the total concentration of amino acids in each recipe. Thus, it was possible that the overgrowth phenotype could be attributed simply to the low total amino acid levels, which differed by 561 mg/L, irrespective of any specific amino acid. To address this concern, I increased the total amino acids of SCD_{ME} (1200 mg/L) to 2000 mg/L to approximate (exceed) the total amino acid levels in SCD_{CSH} (1761 mg/L) without changing the ratios between individual amino acids in SCD_{ME}, designated SCD_{ME}[Up] (recipe in **Appendix 1**). However, even though the total amino acid concentration was high, a particularly strong overgrowth phenotype was observed for Δ*whi*2 and the ESCRT knockout strains (**Fig. 7c**). This implies that the overgrowth phenotype was not affected by the total amino acid concentrations in these experiments. To further rule out effects of total amino acid concentrations as a contributing factor in restricting WT and not the Δ*whi*2 and ESCRT knockout strains, I tested an established recipe with very low total amino acid levels SCD_{Dawes} (810 mg/L,

recipe in **Table 1**), but which has relatively high leucine levels (100 mg/L) [52]. This recipe did not result in overgrowth phenotypes (**Fig. 7d**), despite having ~400 mg/L lower total amino acids than SCD_{ME} (1200 mg/L). This confirms that the total amino acids, at the concentrations tested (between 800 mg/L and 1761 mg/L), do not explain the slow growth of wild type relative to these knockout yeast strains. Together these results are consistent with leucine being sensed in a ratio with other amino acids. Interestingly, the relative amount of leucine in SCD_{Dawes} (12.3% of total) is more comparable to SCD_{CSH} (20.8% of total), while leucine makes up only 2.5% of the total amino acids in SCD_{ME}. Thus, the percent of leucine was important while the absolute amount was not.

The results thus far are consistent with the possibility that either a low leucine threshold exists or that a ratio between leucine and other amino acids in the media regulate the overgrowth phenotype. To address the possibility that the overgrowth phenotype is due to the leucine concentration falling below a threshold, I titrated the leucine concentration in SCD_{CSH} and SCD_{ME} recipes. On modified SCD_{CSH} plates, when the leucine concentration dropped below 60 mg/L and the percent of leucine dropped below 4% of the total amino acid concentration, the overgrowth phenotype returned (**Fig. 8a-d;** recipes in **Appendix 1**). Conversely, increasing the leucine concentration in SCD_{ME} from 30 mg/L to 50 mg/L (4% Leu) or more abolished the overgrowth phenotype (**Fig. 8e-g**). This suggests that rather than the absolute concentration of leucine being the threshold restricting WT strain growth, the overgrowth phenotype occurs when the percent of leucine compared to the total amino acid concentration drops below 4%. This idea is further supported when comparing SCD_{ME}[Up] (48 mg/L, 2% Leu) (**Fig. 9c**) with SCD_{ME}[Leu(50)] (50 mg/L, 4.11% Leu) (**Fig. 9d**), as they have similar leucine

concentrations but different leucine contributions to total amino acid concentration. This is consistent with the threshold of the leucine concentration being 4% of the total amino acid concentration. While not related to the overgrowth phenotype, a drastic increase in overall growth was observed between the SCD_{CSH} media with 30 mg/L of leucine and the one with 40 mg/L of leucine. As an auxotrophic amino acid, it may be possible that there is a minimum threshold of leucine concentration required to sustain growth. However, this seems to indicate that there is a relatively narrow range in which the leucine concentration results in the overgrowth phenotype.

Figure 6

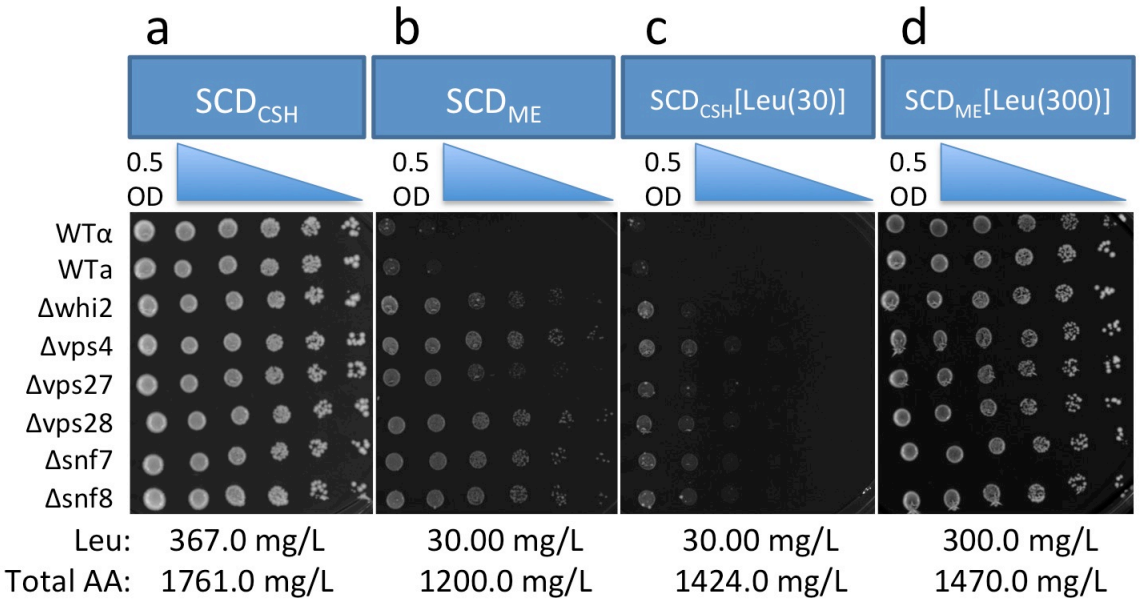


Figure 6 – Low leucine restricts growth in the WT strains, but not the Δ*whi2* or ESCRT complex KO strains

(a) Representative growth of WT, Δ*whi2* and ESCRT knockout strains on high amino acid (SCD$_{CSH}$) medium. All strains grew well at all dilutions. **(b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on low amino acid (SCD$_{ME}$) medium. For WT strains, growth is observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the Δ*whi2* and ESCRT KO strains. **(c)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{CSH}$ with ME levels of leucine. For all strains, growth is reduced, although Δ*whi2* and ESCRT KO strains retain an overgrowth phenotype relative to wild type. **(d)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{ME}$ with high leucine levels. All strains grew well at all dilution factors.

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time. Contrast increased by 20% and brightness increased by 40% for plate **(c)** to improve visibility. Shown is a representative of at least 3 independent experiments using plates generated on 2 different occasions.

Figure 7

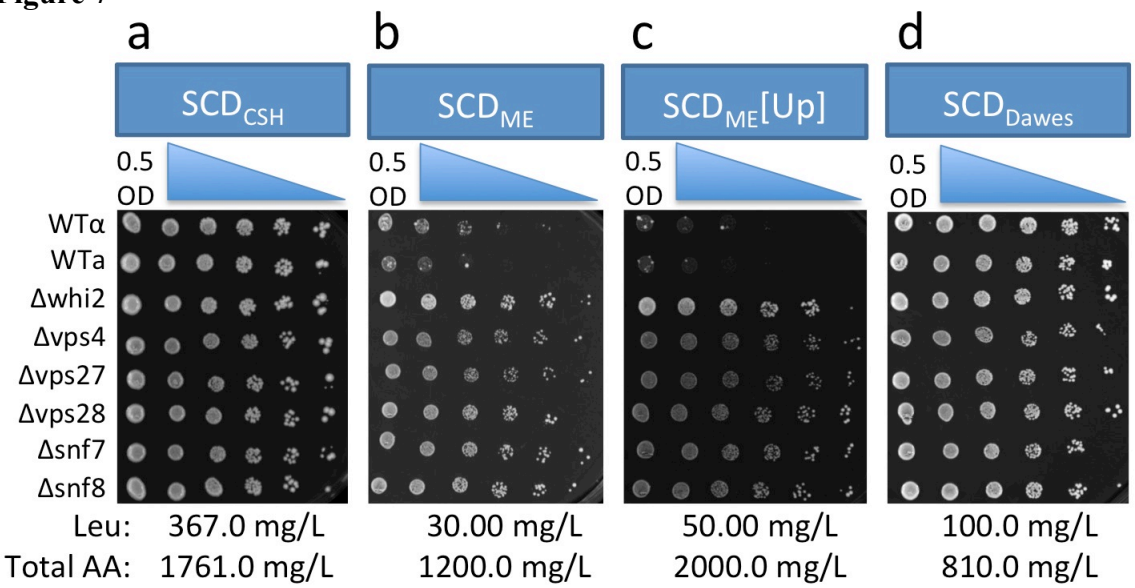


Figure 7 – Overgrowth by Δ*whi2* and ESCRT complex KO strains not dependent on total amino acid concentrations

(a) Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{CSH}$ medium. All strains grew well at all dilution factors. **(b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on published SCD$_{ME}$ medium. WT strains exhibited growth only for the first few dilutions in the series, while growth is observed for all dilution factors for the Δ*whi2* and ESCRT KO strains. **(c)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{ME}$ medium with proportionately higher levels (~1.6? fold) of all amino acid components (2000 mg/L) The growth phenotype of the WT, Δ*whi2* and ESCRT KO on this media reflected SCD$_{ME}$. d) Representative growth of WT, Δ*whi2* and ESCRT KO strains on another established low amino acid, but moderate leucine concentration, (SCD$_{Dawes}$) media. All strains grew well at all dilution factors.

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time. Shown is a representative of at least 3 independent experiments using plates generated on 2 different occasions.

Figure 8 – Leucine contribution of less than 4% by weight to total amino acid concentration may act as threshold of overgrowth phenotype

(a-h) Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD media with varying leucine concentrations as indicated. In **(a-c)**, the WT strains did not grow at all dilution factors, but showed better growth with higher leucine concentrations, while the Δ*whi2* and ESCRT KO strains grew well at all dilutions. In **(d)**, all strains grew well at all dilution factors. **(e-h)** Representative growth of WT, Δ*whi2* and ESCRT KO strains SCD_{ME} media with the indicated leucine concentrations. For any amount of added leucine to SCD_{ME}, growth was observed at all dilution factors for the WT, Δ*whi2* and ESCRT KO strains; no overgrowth phenotypes observed.

Strains were grown in preparation for dilution according to protocol outlined above. All strains for plates **(a)-(d)** were diluted and plated at the same time, and all strains for plates **(e)-(g)** were plated at the same time. Contrast and brightness increased by 40% on plate **(a)** to improve visibility. Shown is a representative of 3 independent experiments using plates generated on 2 different occasions.

Figure 8

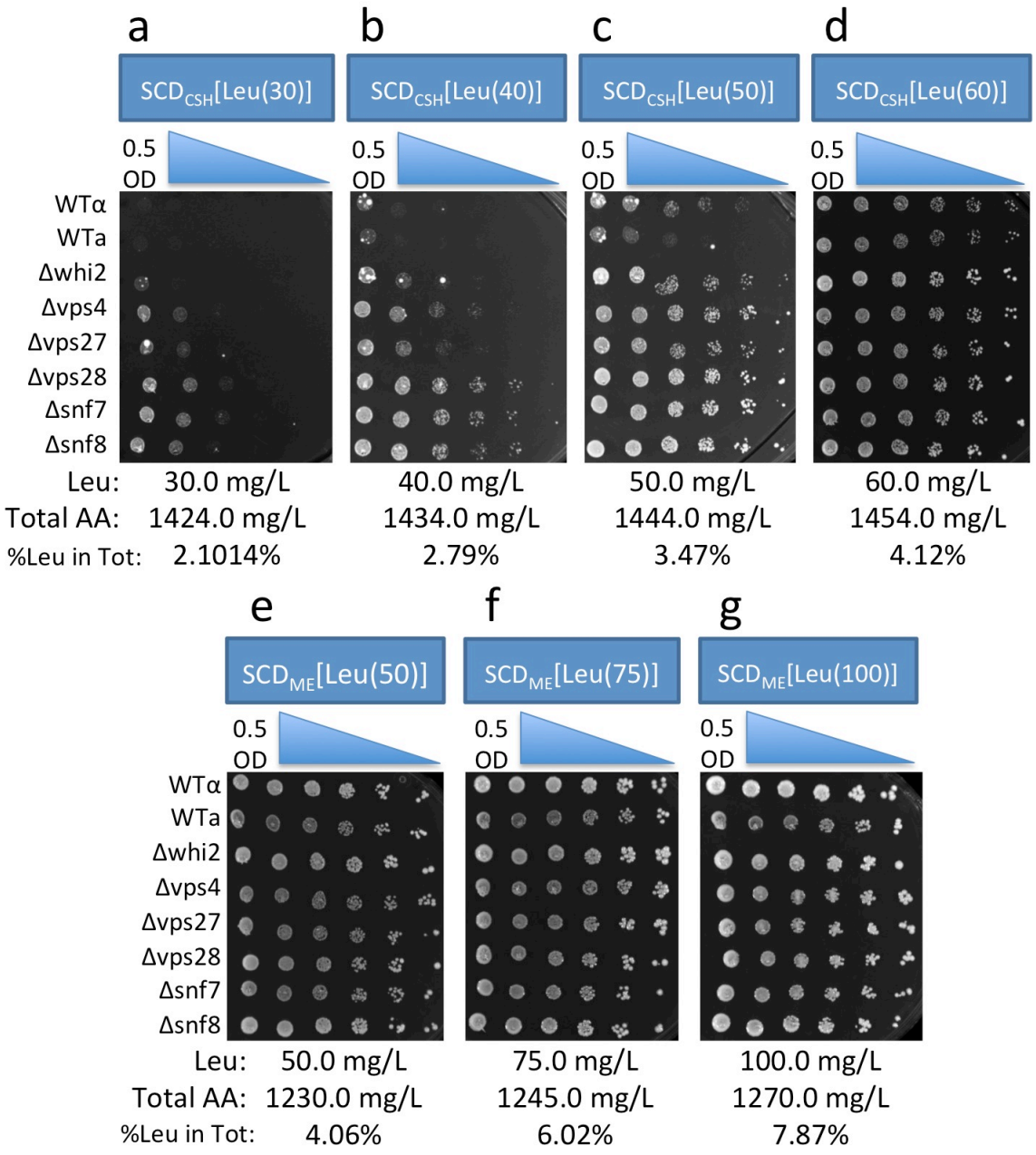

a
b
c
d
SCD$_{CSH}$[Leu(30)]
SCD$_{CSH}$[Leu(40)]
SCD$_{CSH}$[Leu(50)]
SCD$_{CSH}$[Leu(60)]
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 30.0 mg/L 40.0 mg/L 50.0 mg/L 60.0 mg/L
Total AA: 1424.0 mg/L 1434.0 mg/L 1444.0 mg/L 1454.0 mg/L
%Leu in Tot: 2.1014% 2.79% 3.47% 4.12%
e
f
g
SCD$_{ME}$[Leu(50)]
SCD$_{ME}$[Leu(75)]
SCD$_{ME}$[Leu(100)]
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 50.0 mg/L 75.0 mg/L 100.0 mg/L
Total AA: 1230.0 mg/L 1245.0 mg/L 1270.0 mg/L
%Leu in Tot: 4.06% 6.02% 7.87%

Figure 9

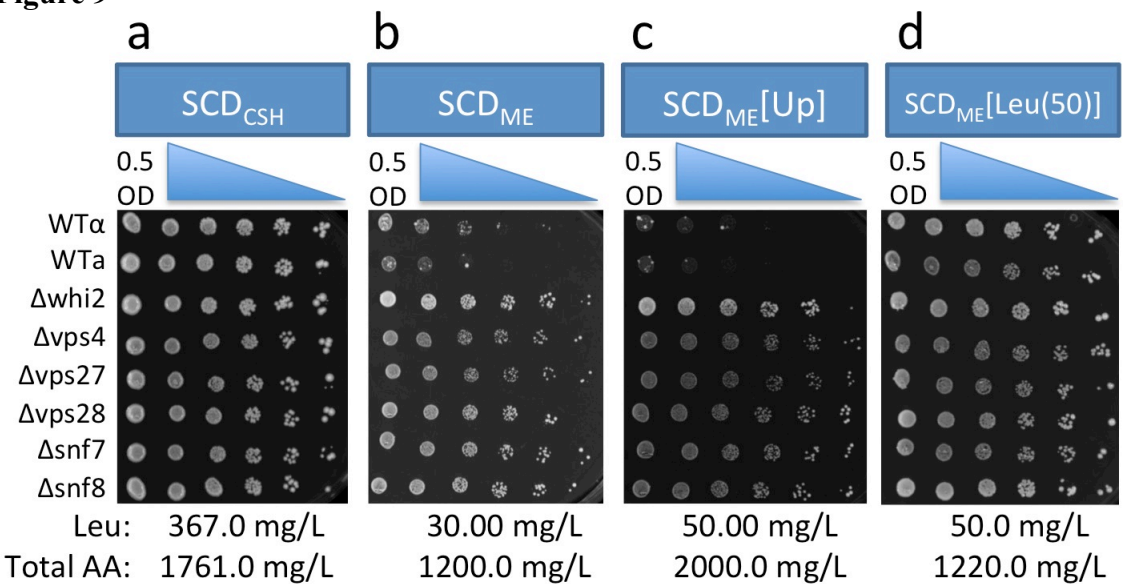


Figure 9 – Low leucine ratio to other amino acids restricts WT growth but not for Δ*whi2* and ESCRT KO strains

(a-c) These are the same panels as **Fig. 7(a-c)** included here for comparison. **(d)** This is reproduced from **Fig. 8e**. Representative growth of WT, Δ*whi2* and ESCRT KO strains on modified SCD_{ME} medium except with slightly higher leucine concentration compared to **(c)**. In contrast to **(b)** and **(c)**, all strains grew well at all dilution factors in **(d)**.

Strains were grown in preparation for dilution according to protocol outlined above. All strains for plates **(a)-(c)** were diluted and plated at the same time, plate **(d)** was plated separately. Shown is a representative of at least 3 independent experiments using plates generated on 2 different occasions.

Other amino acids play a role in regulating growth in low leucine

With the data above suggesting that a specific leucine threshold exists, I wanted to determine if the overgrowth phenotype could be recapitulated in any media comprised of less than 4% by weight of leucine, supporting the hypothesis above that a low leucine environment was determined by less than 4% of leucine contributing to the total amino concentration. To test this, I developed and tested two new low amino acid recipes for overgrowth phenotypes. The first recipe, SCD_{MEDD}, was based on a combination of SCD_{ME} and SCD_{Dawes}, designated SCD_{MEDD} (MEDD – **ME** and **D**awes **D**erived), which has 570 mg/L total amino acids and the same 30 mg/L leucine concentration as SCD_{ME} (5.26% Leu). SCD_{MEDD} resulted in low growth of the WT strains and overgrowth of the ESCRT knockout strains, but interestingly, unlike SCD_{ME}, the Δ*whi2* strain showed WT-like depressed growth (**Fig. 10c**). This dichotomy of Δ*whi2* overgrowth is explored further in below. However, SCD_{MEDD} had a leucine to total amino acid concentration ratio higher than the 4% threshold suggested above, yet exhibited an overgrowth phenotype in the ESCRT knockout strains. This disproves the idea that a threshold of leucine to total amino acid ratio resulted in the overgrowth phenotype, and rather encourages the idea that some ratio between leucine and certain, but not all, amino acids in the recipes was important.

In the second new recipe, SCD_{Aux}, the total amino acid level was reduced even further to 90 mg/L (**Table 2**) and contains only the four auxotrophic amino acids required to sustain viability. SCD_{Aux} has the same leucine concentration as SCD_{ME} of 30 mg/L, but accounts for 33.3% of the total amino acids. The uniform and surprisingly strong growth of the WT, Δ*whi2*, and ESCRT knockout strains on SCD_{Aux} agrees with my earlier results

that neither the low total amino acid concentration nor low absolute leucine concentrations is solely responsible (**Fig. 10d**). As predicted, leucine's 33.3% contribution by weight to total amino acid is above the 4% threshold suggested earlier resulting in no overgrowth phenotype. As this recipe only contained the essential amino acids yet supported robust growth, it was a good system to test whether adjusting the contribution of leucine to the total amino acid concentration could recover the overgrowth phenotype without the potential interference from other amino acids. Even when keeping leucine constant at 30 mg/L and titrating the other auxotrophic amino acids (histidine, lysine and methionine), such that leucine was 2.5% (SCD_{Aux}[2.5% Leu], recipe in **Appendix 1**) of the total amino acids, the overgrowth phenotype was not restored (**Fig. 11d-f**). The overgrowth phenotype on SCD_{MEDD} (5.26% leucine by weight of total amino acids) and the lack of overgrowth on the modified SCD_{Aux}[2.5% Leu] refute the hypothesis made earlier that the percent of leucine in the total amino acid concentration plays a role in the overgrowth phenotype.

These results imply that the overgrowth phenotype is not due to simply the percent of leucine in the total amino acid concentration. As mentioned earlier, the second possibility is that there is a relationship between leucine and a subset of other amino acids that may be the cause of the overgrowth phenotype. This idea is supported by the research done by Sabatini's group showing that mammalian cell growth in response to leucine requires the presence of other amino acids [2]. One amino acid of particular interest is methionine because evidence implicates low methionine concentrations prolong survival in yeast models and mammalian tumor models [52, 53]. Additionally there are lower levels (20 mg/L Met) of methionine in SCD_{Aux} than SCD_{ME} (20 mg/L

Met) or SCD_{CSH} (73.4 mg/L Met). The low methionine concentrations in the SCD_{Aux} media raised the possibility that the lack overgrowth phenotype on SCD_{Aux} may be a result of the methionine concentration affecting survival, as reported in the literature. To test whether the reported methionine restriction increased survival would manifest as overgrowth of the Δ*whi2* and ESCRT KO strains compared to WT strains, I reduced the methionine concentration of SCD_{Aux} to 10 mg/L and also increased it to 100 mg/L. However, my results indicate that increasing methionine to 100 mg/L did not reveal the overgrowth phenotype (**Fig. 12d**). In contrast to the better survival of yeast in low methionine reported in the literature [52, 53], decreasing methionine in SCD_{AUX} to 10 mg/L did not improve growth or survival on the plates, but rather decreased overall growth for all strains (**Fig. 12c**). Such decrease in overall growth could be explained by insufficient methionine, as the lowest methionine concentration used in the literature for methionine restriction is 30 mg/L, even though the SCD_{ME}, SCD_{Dawes}, SCD_{Aux}, and SCD_{MEDD} recipes already only contain 20 mg/L [53]. The inability to recover the overgrowth phenotype when increasing the auxotrophic amino acids together (**Fig. 11d-f**) or methionine individually (**Fig. 12c-d**) in SCD_{AUX} suggests that these auxotrophic amino acids are not sensed in combination with leucine to restrict WT growth that result in the overgrowth phenotype.

The findings suggest that the ratio of leucine to histidine, lysine or methionine are not the individual or in combination the cause of overgrowth phenotype. As the auxotrophic amino acid concentrations between SCD_{Aux} (20 mg/L His, Lys, Met and 30 mg/L Leu) and SCD_{MEDD} (20 mg/L His, Met and 30 mg/L Lys, Leu) are extremely similar, SCD_{MEDD}, but not SCD_{Aux}, contains arginine, aspartic acid, isoleucine,

phenylalanine, threonine, tryptophan, tyrosine, and valine, as well as the purine derivative, adenine, which are the remaining amino acids that would account for the overgrowth phenotype on SCD_{MEDD} that is not present on SCD_{Aux}. To determine which of these amino acids can rescue the overgrowth phenotype, each amino acid was individually added to SCD_{Aux} at the same concentrations present in SCD_{MEDD}. However, adding back the amino acids or adenine individually did not rescue the overgrowth phenotype (**Fig. 13a-h**). These results indicate that a single amino acid addition is not sufficient to rescue the overgrowth phenotype on SCD_{Aux}, which implies that a group of amino acids are required to restrict growth in the low leucine of SCD_{Aux}. This idea is not novel, as studies in *S. cerevisiae* have shown that specific amino acids are considered "high-end" nitrogen sources, which promote growth, while others were considered "low-end" nitrogen sources, which have no positive effect on growth [55]. I also tested the following combinations of amino acids added to SCD_{Aux}: arginine, asparagine, valine, and threonine; isoleucine, phenylalanine, tryptophan, and tyrosine; valine, tryptophan, and tyrosine; arginine, asparagine, isoleucine, phenylalanine, and threonine; arginine, asparagine, tryptophan, and tyrosine; isoleucine, phenylalanine, and valine; and lastly, tryptophan, threonine, and tyrosine (recipes in **Appendix 1**). However, none of these combinations resulted in the restriction of WT growth and overgrowth of the Δ*whi2* or ESCRT knockout strains (**Fig 14a-g**). As not all possible amino acid combinations have been tested, and the classification of "high-end" and "low-end" nitrogen sources were not considered, a definitive conclusion cannot be made at this time.

Figure 10

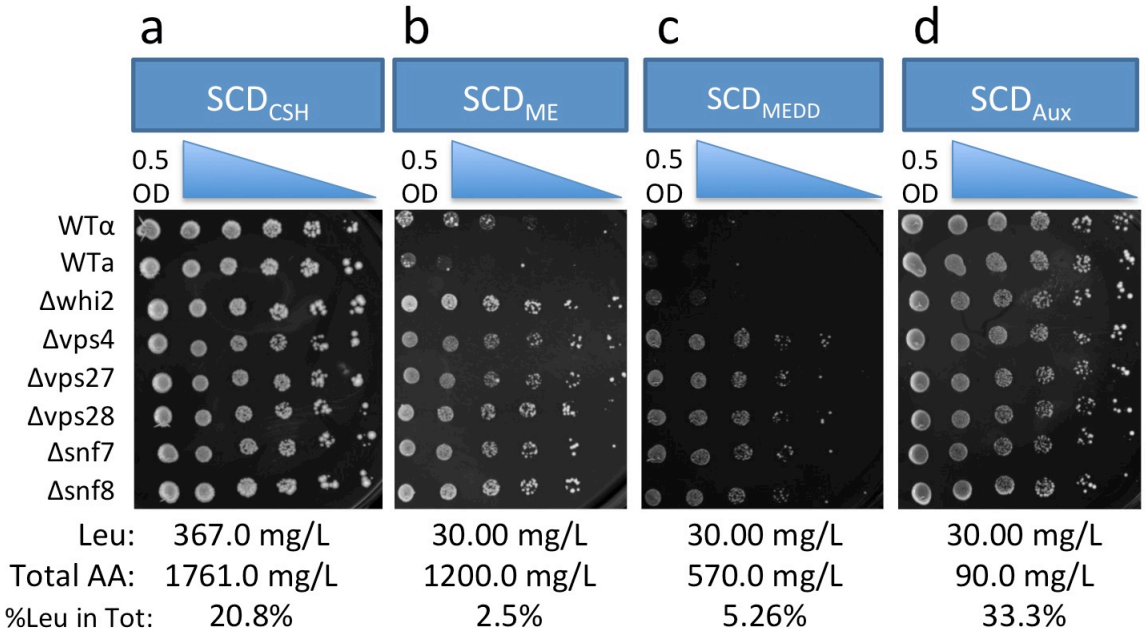


Figure 10 – Leucine is sensed in a ratio with other amino acids even at low concentrations

(a) Representative growth of WT, Δ*whi2* and ESCRT KO strains on rich (SCD$_{CSH}$) media. All strains grew well at all dilution factors. **(b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{ME}$ medium. For WT strains, growth is observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the Δ*whi2* and ESCRT KO strains. **(c)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{MEDD}$ medium. For WT and Δ*whi2* strains, growth is faintly observed only for the first few dilutions in the series, while growth is observed for all dilutions factors for the ESCRT KO strains. By visual comparison of spot density, overall growth on SCD$_{MEDD}$ is slightly poorer than SCD$_{ME}$. **(d)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on medium containing only the 4 auxotrophic amino acids, SCD$_{Aux}$. All strains grew well at all dilution factors. By visual comparison of spot density, overall growth is slightly worse than SCD$_{CSH}$.

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time. Shown is a representative of at least 3 independent experiments using plates generated on 2 different occasions.

Figure 11 – Leucine concentration independent of other amino acids, does not account for overgrowth phenotype

a) Representative growth of WT, Δ*whi2* and ESCRT KO strains on rich (SCD_{CSH}) media. All strains grew well at all dilution factors. **b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on pre-established low nutrient (SCD_{ME}) media. For WT strains, growth is observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the Δ*whi2* and ESCRT KO strains. **c)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD_{Aux}. All strains grew well at all dilution factors. By visual comparison of spot density, overall growth is slightly worse than SCDCSH. **d-f)** Representative growth of WT, Δ*whi2* and ESCRT KO strains when varying amino acids aside from leucine in the auxotrophic amino acid media (SCD_{Aux}). Varying the other amino acids while keeping leucine constant did not result in the recovery of the overgrowth phenotype.

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time. Shown is a representative of at least 3 independent experiments using plates generated on 2 different occasions.

Figure 11

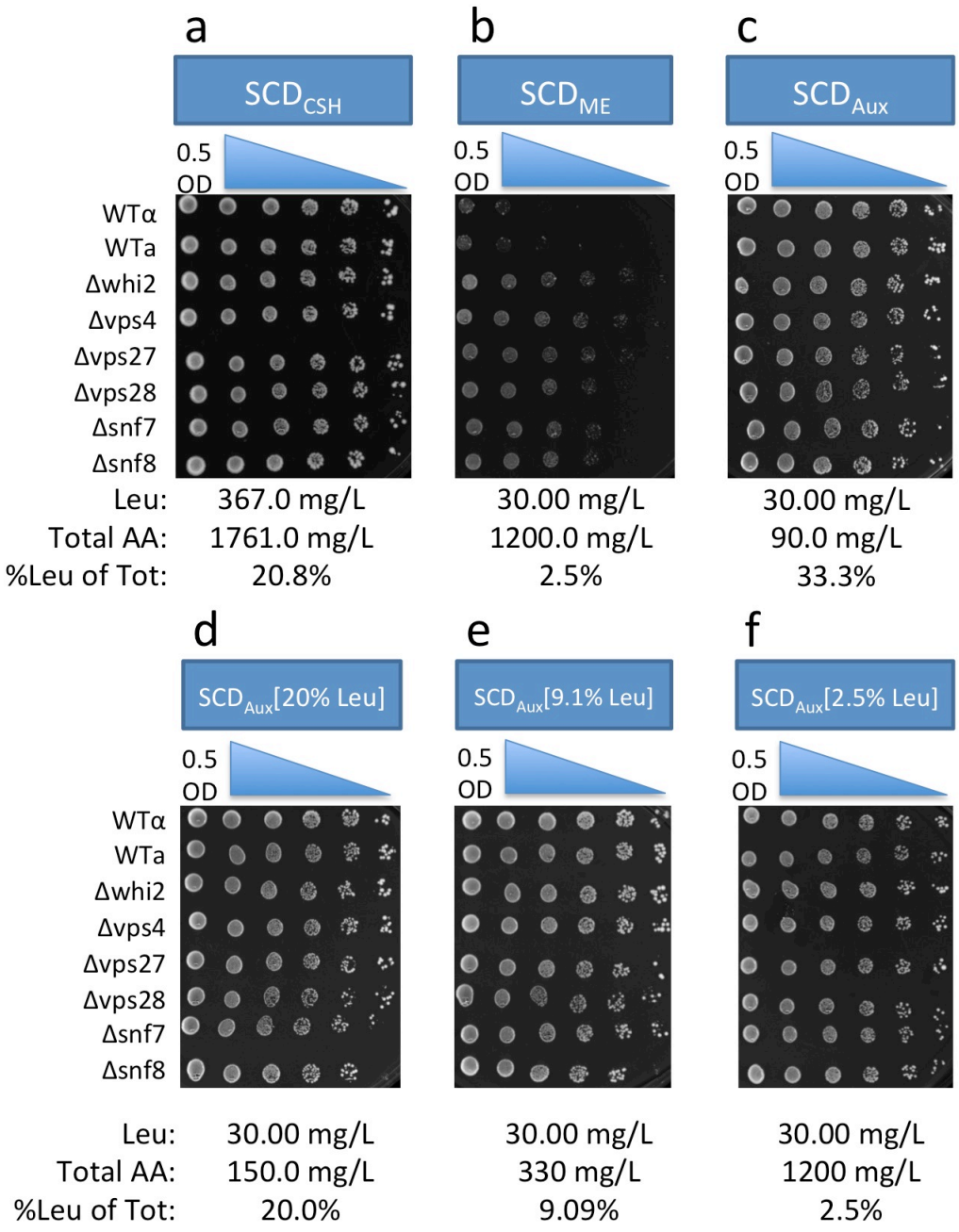

a
SCD CSH
b
SCD ME
c
SCD Aux
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 367.0 mg/L 30.00 mg/L 30.00 mg/L
Total AA: 1761.0 mg/L 1200.0 mg/L 90.0 mg/L
%Leu of Tot: 20.8% 2.5% 33.3%
d
SCD Aux[20% Leu]
e
SCD Aux[9.1% Leu]
f
SCD Aux[2.5% Leu]
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 30.00 mg/L 30.00 mg/L 30.00 mg/L
Total AA: 150.0 mg/L 330 mg/L 1200 mg/L
%Leu of Tot: 20.0% 9.09% 2.5%

Figure 12

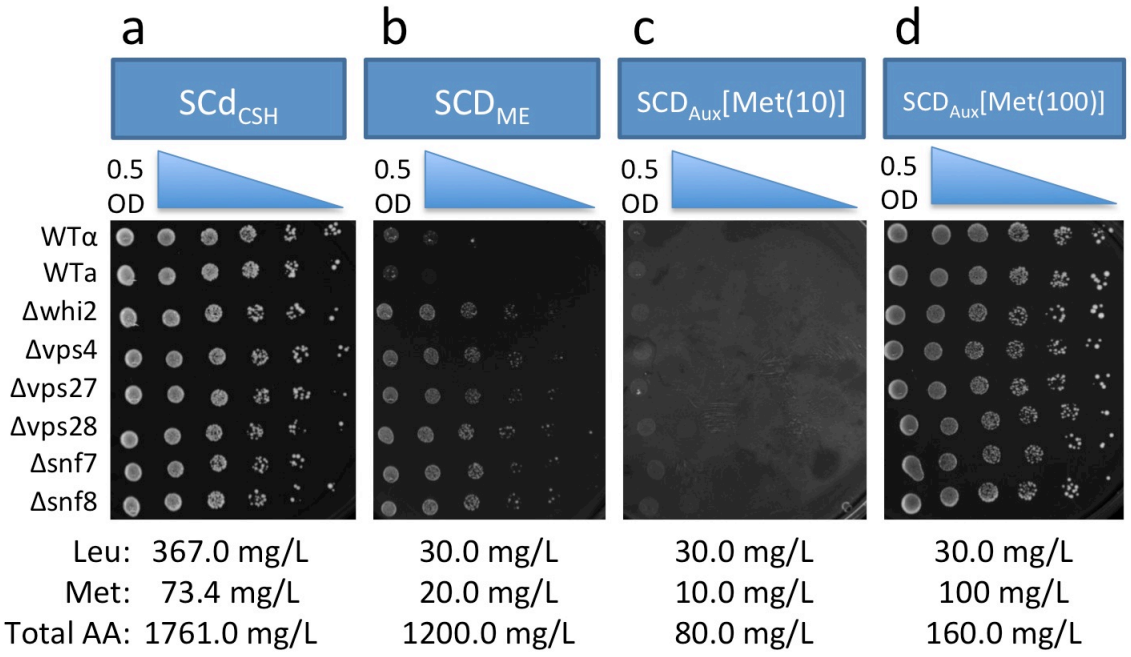


Figure 12 – Overgrowth phenotype by *whi2* and ESCRT mutants is not due to leucine being an auxotrophic amino acid

(a) Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{CSH}$ medium. All strains grew well at all dilution factors. **(b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD$_{ME}$ media. For WT strains, growth is observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the Δ*whi2* and ESCRT KO strains. **(c-d)** Representative growth of WT, Δ*whi2* and ESCRT KO strains when varying methionine concentrations in the context of the auxotrophic amino acid media SCD$_{Aux}$. Due to the requirement for methionine, decreasing methionine to 10 mg/L resulted in stunted overall growth for all strains in **(c)**. The poor growth made differentiating growth phenotype impossible, and so it is unknown if an overgrowth was present. **(d)** Increasing the amount of methionine did not change the growth phenotype for any of the strains.

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time. Contrast and brightness were increased by 40% for plate **(c)** to improve visibility. Shown is a representative of at least 2 independent experiments using plates generated on 2 different occasions.

Figure 13

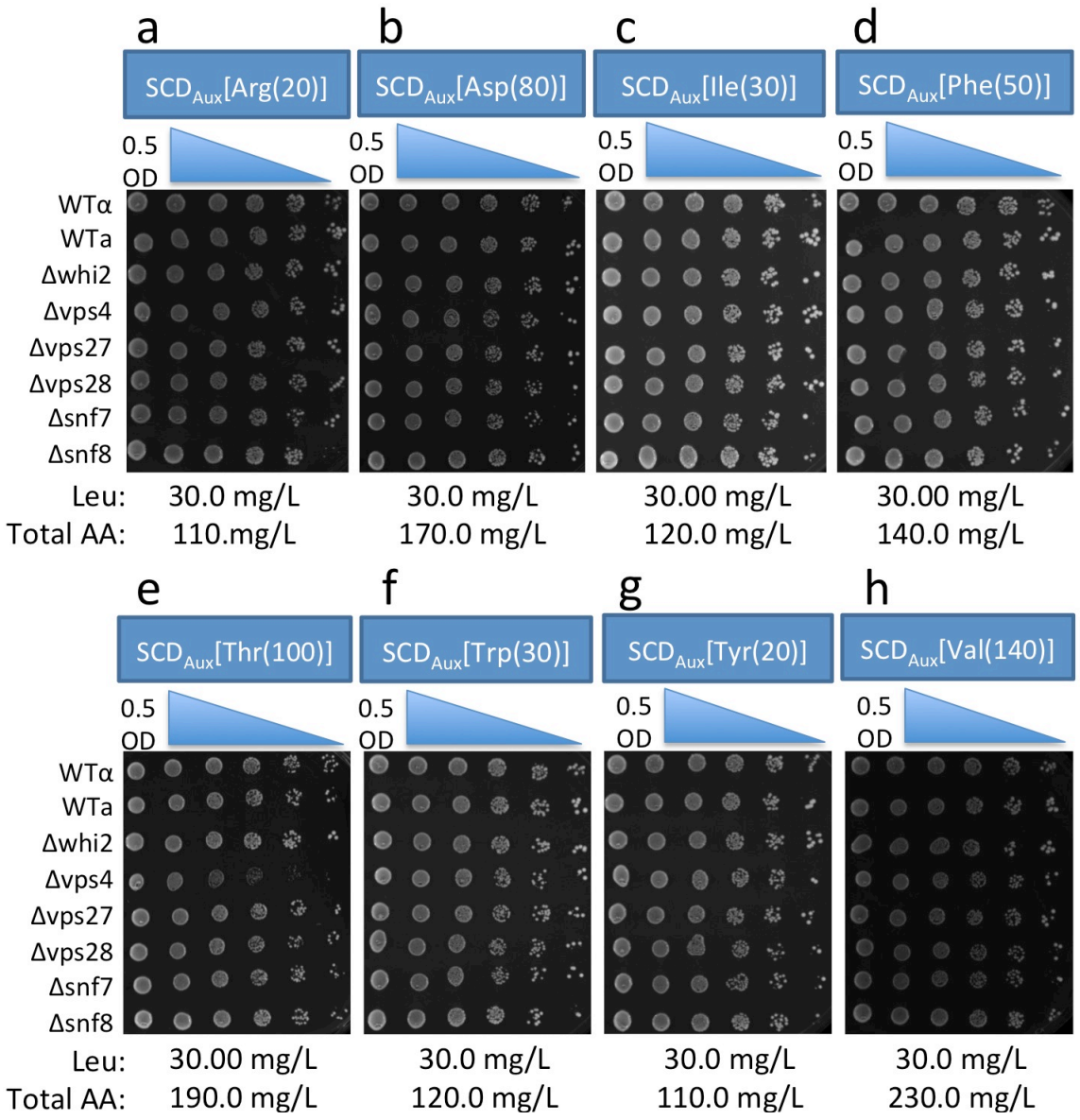


Figure 13 – Low leucine overgrowth phenotype for the Δ*whi2* or ESCRT complex KO strain requires more than leucine and a single amino acid addition

(b-h) Representative growth of WT, Δ*whi2* and ESCRT KO strains on modified SCD_{Aux}, where arginine **(b)**, aspartic acid **(c)**, isoleucine **(d)**, phenylalanine **(e)**, threonine **(f)**, tryptophan **(g)**, or valine **(h)** were individually added to SCD_{Aux} to reflect SCD_{MEDD} levels. Regardless of the individual amino acid added, the WT, Δ*whi2* and ESCRT KO strains grew well at all dilution factors.

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time. Shown is a representative of 3 independent experiments using plates generated on 2 different occasions.

Figure 14

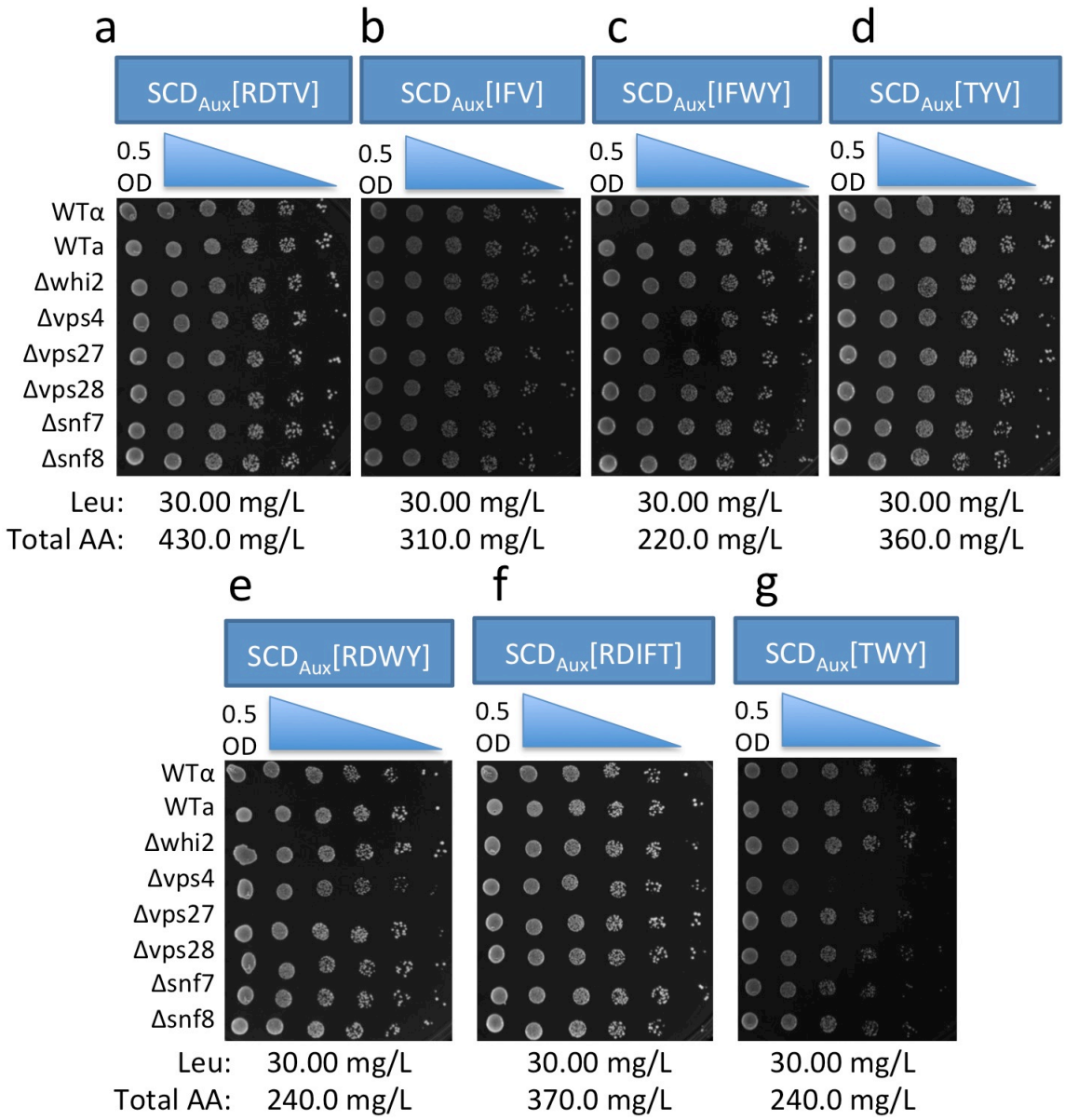


Figure 14 – Adding back combinations of amino acids have yet to reveal overgrowth phenotype on SCD$_{Aux}$

(a-g) Representative growth of WT, Δ*whi2* and ESCRT KO strains on modified SCD$_{Aux}$ where combinations of amino acids were added to SCD$_{MEDD}$ levels. Combinations of amino acids were randomly determined. No overgrowth of the Δ*whi2* and ESCRT KO strains was observed for any combination tested. **(g)** Δ*vps4* showed reduced growth.

Strains were grown in preparation for dilution according to protocol outlined above. Strains for plates **(a), (c), (d), (f)** were diluted and plated on the same day, and strains for **(b), (e), (g)** were diluted and plated on the same day. Contrast increased by 20% and brightness increased by 40% for plate **(b)** to improve visibility. Shown is a representative of 2 experiments using plates generated on 1 occasion.

Chapter 4

Glutamic acid is sufficient to rescue Δ*whi2* strain overgrowth on SCD_{MEDD}

Results

The restricted growth of the Δ*whi2* strain on the SCD_{MEDD} plate compared to on SCD_{ME} provides a strategy to explore the role that *WHI2* plays in restricting growth in low leucine environments. Discovering the differences between SCD_{MEDD} and SCD_{ME} that rescue the overgrowth phenotype in the Δ*whi2* strain could provide insight into the function that *WHI2* plays in nutrient sensing in low leucine environments.

The biggest difference between SCD_{MEDD} and SCD_{ME} is the lack of serine and glutamic acid in SCD_{MEDD}, but there are also a lower concentrations of threonine (100 mg/L in SCD_{MEDD}, 200 mg/L in SCD_{ME}), aspartic acid (80 mg/L in SCD_{MEDD}, 100 mg/L in SCD_{ME}), and valine (140 mg/L in SCD_{MEDD}, 150 mg/L in SCD_{ME}) (recipe in **Table 2**). To elucidate which of these amino acids differences contribute to the Δ*whi2* overgrowth observed in SCD_{ME}, the amino acids were added to SCD_{MEDD} to restore SCD_{ME} levels to assess their effect on the Δ*whi2* growth. When restoring all five amino acid differences (**Fig. 15c**) or even the three amino acids with the largest concentration difference (serine, threonine, and glutamic acid) (**Fig. 15d**) to the SCD_{MEDD} media, Δ*whi2* strain restored the overgrowth phenotype on SCD_{ME}. This indicates that serine, threonine, and glutamic acid, either together or independently, can restore the overgrowth of the Δ*whi2* strain in low leucine conditions. To determine if this dramatic effect on growth was the property

of any one of the three amino acids sufficient to rescue the Δ*whi2* overgrowth, each was tested separately in SCD_{MEDD} (recipe in **Table 2**). When testing these amino acids individually, glutamic acid was necessary and sufficient to rescue the phenotype (**Fig. 15e**), while adding back threonine or serine individually did not rescue the Δ*whi2* overgrowth (**Fig. 16g, 16h**). 100 mg/L of the remaining 6 amino acids not present in either SCD_{MEDD} or SCD_{ME} (alanine, asparagine, cysteine, glycine, glutamine, and proline) were also individually added to SCD_{MEDD} to determine whether glutamic acid is unique in rescuing the Δ*whi2* strain overgrowth. However, none of the amino acids tested displayed the same ability as glutamic acid in rescuing the Δ*whi2* strain overgrowth (**Fig. 16a-f**).

The addition of glutamic acid to SCD_{MEDD} led to changes in the recipe, e.g. the total amino acid concentration or pH of media, that needed to be further addressed. To address the changes in total amino acid concentration, I increased the total amino acids of SCD_{MEDD} (570 mg/L) to reflect SCD_{MEDD}[Glu(100)] (670 mg/L) without changing the ratio between each amino acid in SCD_{MEDD}. However, this modified SCD_{MEDD} recipe (SCD_{MEDD}[670 tot]) with higher total amino acid concentration did not affect the Δ*whi2* strain growth on SCD_{MEDD} (**Fig. 17c**). The lack of effect that the higher total amino acid had in Δ*whi2* overgrowth phenotype was reconfirmed when testing the effect of alanine, asparagine, cysteine, glycine, glutamine, and proline on SCD_{MEDD}, which all also had a 670 mg/L total amino concentration (**Fig. 16a-f**). To rule out the possibility that acidity was a growth stimulant in the Δ*whi2* strain, 100 mg/L of aspartic acid was added to SCD_{MEDD} media to reflect the amount of glutamic acid added to SCD_{MEDD}[Glu(100)]. The addition of aspartic acid to SCD_{MEDD} failed to rescue the Δ*whi2* overgrowth

phenotype (**Fig. 17d**), indicating that glutamic acid is affecting Δ*whi2* growth impendent of its acidic characteristics.

As leucine has been shown earlier to play a role in the overgrowth phenotype, 100 mg/L leucine was added to SCD_{MEDD} to test whether leucine would affect Δ*whi2* growth on SCD_{MEDD}. While increasing leucine rescued Δ*whi2* growth (**Fig. 17e**), the growth of the WT and ESCRT KO strains reflected SCD_{CSH}, suggesting the overgrowth phenotype on SCD_{MEDD}[Glu(100)] was abolished by adding additional leucine.

While glutamic acid was identified as being necessary and sufficient in rescuing Δ*whi2* overgrowth in low leucine, there is the possibility that this is not a *WHI2*-specific effect. This is especially true due to the possibility of secondary mutations may arise in a gene KO strain, but is unlikely in this circumstance as the substrain used had been verified to lack secondary mutations [34]. However, to verify that the Δ*whi2* overgrowth on SCD_{MEDD}[Glu(100)] and SCD_{ME} is *WHI2* specific, a plasmid encoding HA-Whi2 regulated by a constitutive promoter (PGK promoter) was transformed into a Δ*whi2* strain and grown on SCD_{MEDD}[Glu(100)] and SCD_{ME}. The *whi2*-rescued strain grew similarly to the WT strains transformed with empty vectors, on SCD_{ME} and SCD_{MEDD}[Glu(100)] (**Fig. 19c, 19d**). This indicates that *WHI2* plays an important role in sensing the low leucine environment of SCD_{ME} and SCD_{MEDD}. However, this further implies a role of *WHI2* in a glutamate pathway, which may be important in regulating growth in low leucine conditions.

Figure 15 – *WHI2* but not most ESCRTs are required to respond to low levels of the non-essential glutamic acid

(a) Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD_{MEDD}. For WT and Δ*whi2* strains, growth is faintly observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the ESCRT KO strains. (**b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on pre-established low nutrient (SCD_{ME}) media. For WT strains, growth is observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the Δ*whi2* and ESCRT KO strains. (**b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD_{MEDD} with amino acids added back so SCD_{MEDD} to reflect SCD_{ME}. The growth phenotype reflected that observed on SCD_{ME}; growth of WT strains is observed only for the first few dilutions in the series, while growth is observed for most dilution factors for the Δ*whi2* and *Δvps4* strains, and for all dilution factors for the remaining ESCRT KO strains. Amino acids in (c) and **(**d) appear to suppress growth compared to (e). (**e)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD_{MEDD} with glutamic acid added back to reflect SCD_{ME} levels. The growth phenotype reflected that observed on SCD_{ME}; growth of WT strains is observed only for the first few dilutions in the series, while growth is observed for most dilution factors for the Δ*whi2* and *Δvps4* strains, and for all dilution factors for the remaining ESCRT KO strains.

Strains were grown in preparation for dilution according to protocol outlined above. Strains for plates **(a)-(d)** were diluted and plated at the same time. Shown is a representative of at least 3 independent experiments using plates generated on at least one different occasion.

Figure 15

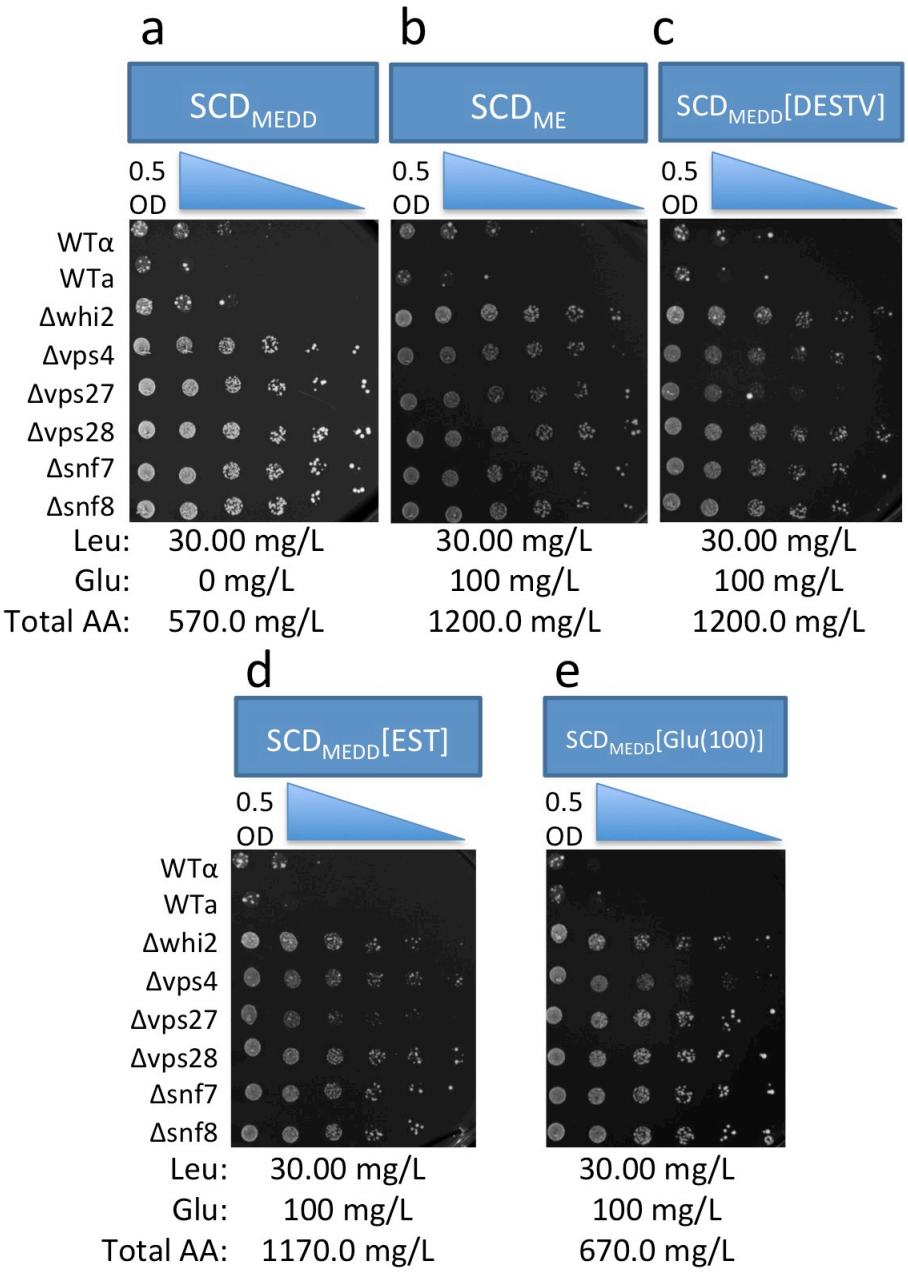

a
b
c
SCD$_{MEDD}$
SCD$_{ME}$
SCD$_{MEDD}$[DESTV]
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 30.00 mg/L 30.00 mg/L 30.00 mg/L
Glu: 0 mg/L 100 mg/L 100 mg/L
Total AA: 570.0 mg/L 1200.0 mg/L 1200.0 mg/L
d
e
SCD$_{MEDD}$[EST]
SCD$_{MEDD}$[Glu(100)]
Leu: 30.00 mg/L 30.00 mg/L
Glu: 100 mg/L 100 mg/L
Total AA: 1170.0 mg/L 670.0 mg/L

Figure 16 – Glutamic acid is the only amino acid that has an effect on the difference in *Δwhi2* overgrowth between SCD_{ME} and SCD_{MEDD}

(a-h) Representative growth of WT, Δ*whi2* and ESCRT KO strains on modified SCD_{MEDD} media, where 100 mg/L of individual amino acids not present in SCD_{MEDD} were added back. Growth phenotypes reflected SCD_{MEDD} regardless of any single amino acid added. The ESCRT KOs showed growth for all dilution factors, while the WT and Δ*whi2* strains only showed weak growth at the first dilution factors. In (e) and (f), glycine and proline appear to result very slight improvement of growth in Δ*whi2* compared to WT. This may be attributable to growth from breakthrough colonies.

Strains were grown in preparation for dilution according to protocol outlined above. All strains for plates **(a), (b)** and **(e)-(h)** were diluted and plated at the same time. Strains for **(c)** and **(d)** were diluted and plated at separate times from each other and the rest of the plates. Contrast increased by 20% and brightness increased by 40% for plate **(c)** to improve visibility. Shown is a representative of 3 independent experiments using plates generated on 2 different occasions.

Figure 16

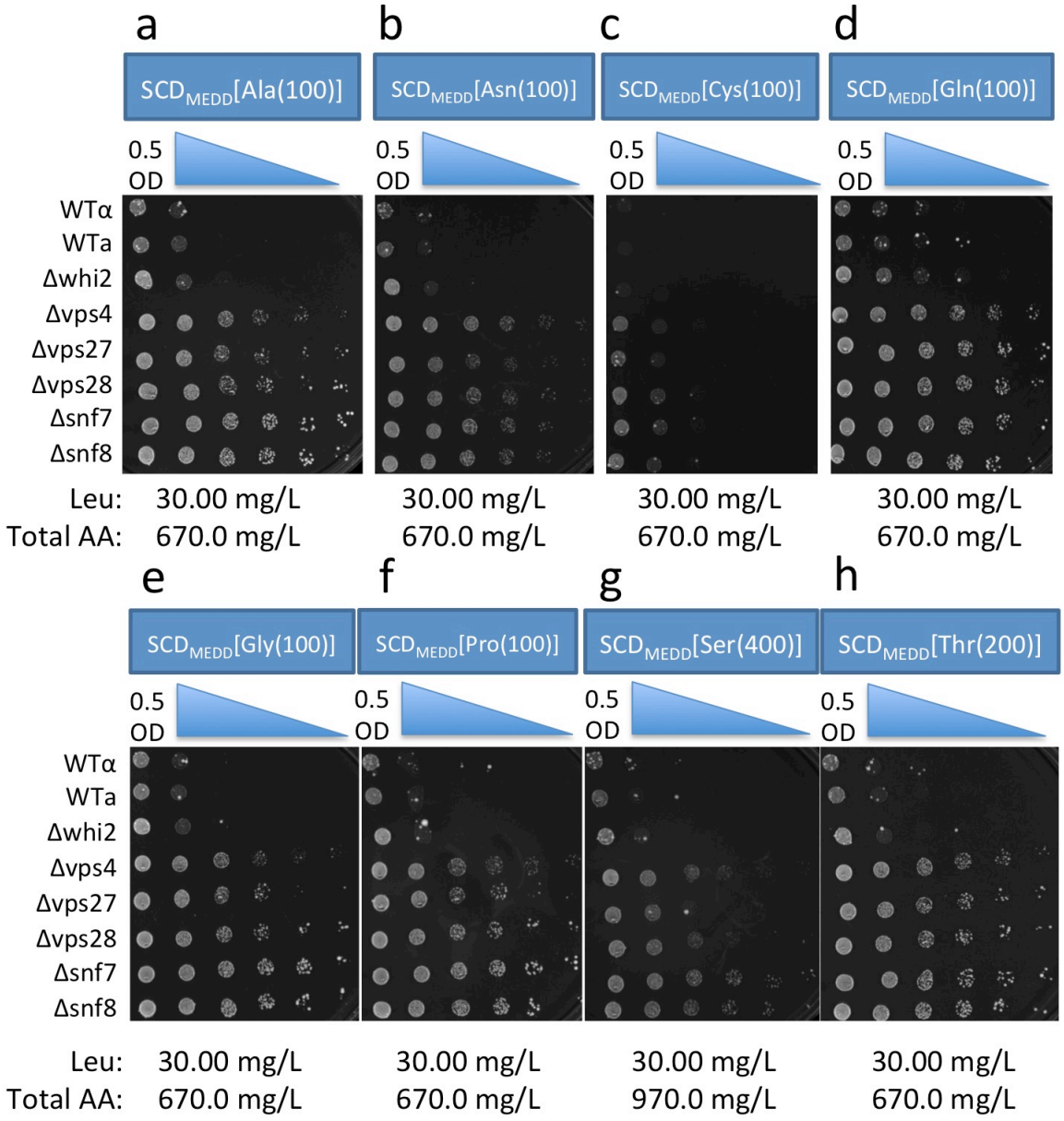

a
b
c
d
SCD MEDD [Ala(100)]
SCD MEDD [Asn(100)]
SCD MEDD [Cys(100)]
SCD MEDD [Gln(100)]
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 30.00 mg/L 30.00 mg/L 30.00 mg/L 30.00 mg/L
Total AA: 670.0 mg/L 670.0 mg/L 670.0 mg/L 670.0 mg/L
e
f
g
h
SCD MEDD [Gly(100)]
SCD MEDD [Pro(100)]
SCD MEDD [Ser(400)]
SCD MEDD [Thr(200)]
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 30.00 mg/L 30.00 mg/L 30.00 mg/L 30.00 mg/L
Total AA: 670.0 mg/L 670.0 mg/L 970.0 mg/L 670.0 mg/L

Figure 17 – Other changes to SCD_{MEDD} when adding glutamic acid does not rescue Δ*whi2* overgrowth

(a) Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD_{MEDD}. For WT and Δ*whi2* strains, growth is faintly observed only for the first few dilutions in the series, while growth is observed for all dilutions factors for the ESCRT KO strains. **(b)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD_{MEDD} with 100 mg/L added glutamic acid. WT, Δ*whi2* and ESCRT KO growth represented SCD_{ME}; the Δ*whi2* and ESCRT KO strains had an overgrowth phenotype compared to WT. **(c)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on modified SCD_{MEDD} that retained the same relative concentrations of amino acids, but increased the total amino acids to 670 mg/L. Growth for all strains reflected what was observed on SCD_{MEDD}. **(d)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on modified SCD_{MEDD}, where 100 mg/L of aspartic acid was added. Growth for all strains reflected what was observed on SCD_{MEDD}. **(e)** Representative growth of WT, Δ*whi2* and ESCRT KO strains on SCD_{MEDD} with added leucine. WT, Δ*whi2* and ESCRT KO strains all showed growth for every dilution factor.

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time except for plate **(c)**. Shown is a representative of at least 3 independent experiments using plates generated on 2 different occasions.

Figure 17:

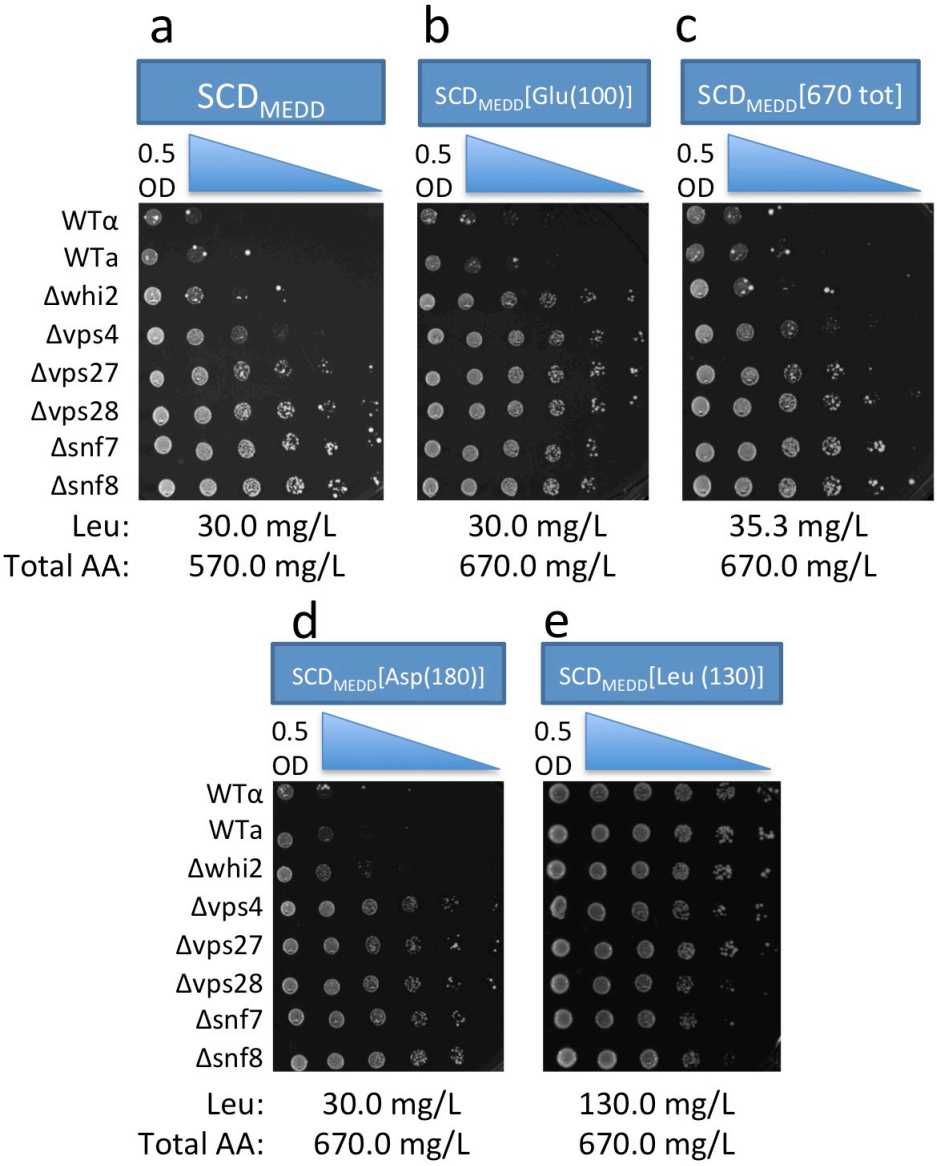

a
b
c
SCD MEDD
SCD MEDD[Glu(100)]
SCD MEDD[670 tot]
0.5 OD
WTα
WTa
Δwhi2
Δvps4
Δvps27
Δvps28
Δsnf7
Δsnf8
Leu: 30.0 mg/L 30.0 mg/L 35.3 mg/L
Total AA: 570.0 mg/L 670.0 mg/L 670.0 mg/L
d
e
SCD MEDD[Asp(180)]
SCD MEDD[Leu (130)]
Leu: 30.0 mg/L 130.0 mg/L
Total AA: 670.0 mg/L 670.0 mg/L

Figure 18

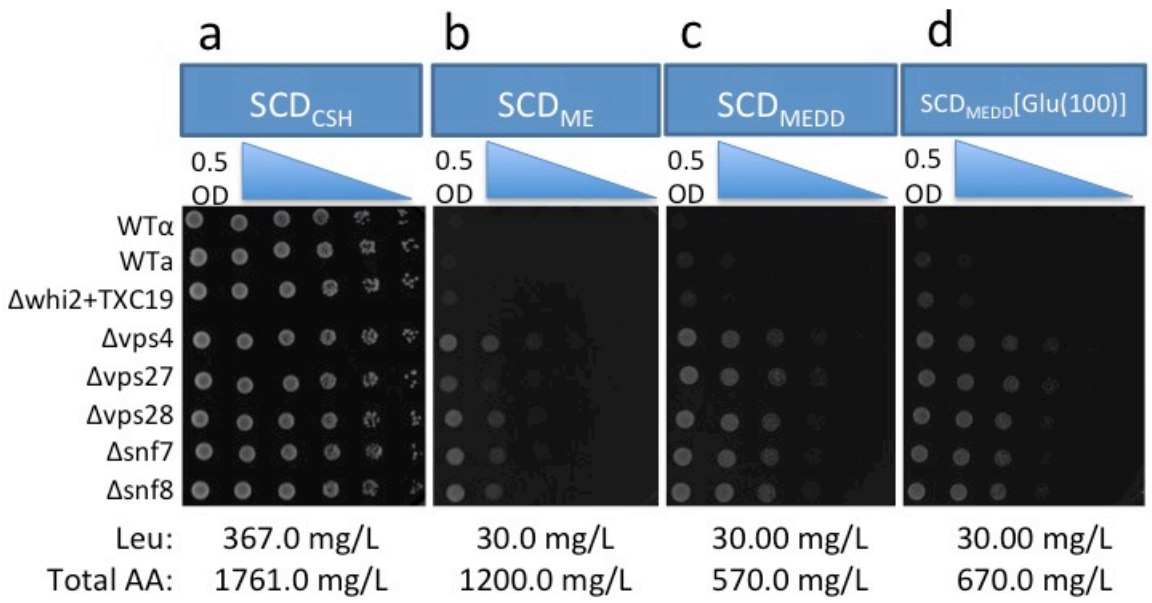


Figure 18 – *WHI2* complementation in the Δ*whi2* strain restores WT-like growth phenotype in low leucine conditions

Δ*whi2* was transformed with TXC19, a plasmid containing constitutively expressed HA-Whi2. The WT and ESCRT KO strains were transformed with BQ23, an empty vector with the same selection marker as TXC19. Protocol can be found in text.

(a) Representative growth of transformed WT, Δ*whi2* and ESCRT KO strains on SCD$_{CSH}$ medium. All strains grew well at all dilution factors. **(b)** Representative growth of transformed WT, Δ*whi2* and ESCRT KO strains on SCD$_{ME}$ media. For WT strains and the complemented Δ*whi2 strain,* growth is observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the ESCRT KO strains. Complementation of *whi2* abolished the overgrowth observed in 6(b). **(c)** Representative growth of transformed WT, Δ*whi2* and ESCRT KO strains on SCD$_{MEDD}$. For transformed WT strains and complemented Δ*whi2*, growth is faintly observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the ESCRT KO strains. **(d)** Representative growth of transformed WT, Δ*whi2* and ESCRT KO strains on SCD$_{MEDD}$ with added glutamic acid. For transformed WT strains and complemented Δ*whi2*, growth is observed only for the first few dilutions in the series, while growth is observed for all dilution factors for the ESCRT KO strains. Complementation of *whi2* abolished the overgrowth observed in 15(e).

Strains were grown in preparation for dilution according to protocol outlined above. All strains were diluted and plated at the same time. Contrast increased by 20% and brightness increased by 40% for plate **(b), (c), (d)** to improve visibility. Shown is a representative of 3 independent experiments using plates generated once.

Chapter 5

Discussion Of Results and Future Directions

Discussion of Results

Utilizing different amino acid conditions revealed two significant conclusions about the *Δwhi2* overgrowth phenotype observed for a number of yeast knockout strains in specific low nutrient conditions. First, it was identified that leucine is an important amino acid in regulating growth, which is not appropriately regulated in low leucine conditions without the ESCRT complex or *WHI2*. It was further shown that other amino acids are required to restrict WT growth in low leucine, implying that other amino acids are sensed along with leucine to regulate cellular growth. Additionally, it was shown that glutamic acid is the only amino acid that can rescue the overgrowth of the *Δwhi2* strain on SCD_{MEDD}, implying that *WHI2* may play a role in glutamic acid synthesis in stress conditions. Lastly, the growth of the *Δwhi2* strain on SCD_{MEDD} and SCD_{ME} was restored to WT levels after transformation with HA-Whi2, indicating that *WHI2* plays a significant role in regulating cellular growth.

It is interesting to note that the many different SCD recipes are utilized in the field and most papers do not specify the exact components. While certain recipes, such as SCD_{CSH}, SCD_{ME}, and SCD_{Dawes}, are more commonly used, they are not well characterized or classified. It becomes more convoluted as different groups make modifications to the recipes to suit the amino acid(s) they are investigating. With my results indicating that certain subsets of amino acids are sensed together to regulate

growth, the results obtained when changing media components may not be strictly indicative of the effect of a single amino acid, but rather a subset of amino acid interactions. However, without knowing which amino acids are sensed together, much more needs to be done before the effects of different SCD recipes can be understood.

While I showed that other amino acids play an important role in restricting growth in response to low leucine, it remains unclear specifically which amino acids have this function. One aspect yet to be tested is the contribution of these amino acids to the metabolizable food source, not just as signaling molecules. The Hall group showed in *S. cerevisiae* that "high-end" amino acids arginine, asparagine, and glutamine had a potent positive effect on S6 phosphorylation, while serine, threonine, isoleucine, leucine, valine, aspartate, glutamate, alanine, methionine, phenylalanine, and tryptophan individually resulted in rapid depression of S6 phosphorylation, and were designated "low-end" amino acids [55]. This is corroborated by some of the amino acid additions to the different SCD media that I have tested here, such as slightly depressed overall growth was observed after supplementing SCD_{MEDD} with serine (**Fig. 15g**). One possibility to consider could be the balance of "high-end" or "low-end" amino acids with leucine. For example, an inadequate amount of "high-end" amino acids or excessive "low-end" amino acids relative to leucine could result in the unfavorable growth condition that is not sensed by Δ*whi2* and ESCRT knockout strains. However, one caveat to the data provided by the Hall group in classification of some amino acids, especially leucine, as "low-end", when it has been shown in our *S. cerevisiae* model and in mammalian cells to have a potent positive effect on growth. One of the reasons for this contradiction could be due to the auxotrophic requirements of leucine in our yeast model and mammalian cells that does

not seem to be present in the yeast model the Hall group uses. While the idea between a balance of "high-end" and "low-end" amino acids needs to be tested, it would be beneficial to reclassify the "high-end" and "low-end" amino acids according to Hall and colleagues for our *S. cerevisiae* model.

The Huang group reported that ratios between sets of amino acids regulates growth, consistent with my findings that ,The Huang group also reported findings supporting the idea that ratios between sets of amino acids regulates growth [56]. Using the same *S. cerevisiae*, the group showed that the ratio between the auxotrophic amino acids, histidine, leucine, and lysine, and the non-auxotrophic amino acids greatly affected the chronological lifespan of yeast [56]. Additionally, this study concluded that glutamic acid in extended the lifespan of *S. cerevisiae*, which they attributed to the enhancement of ROS (reactive oxygen species) stress resistance [57]. In a glutamate dehydrogenase knockout model, glutamate addition was shown to suppress the production of ROS [57].

My observation that the unique ability for glutamic acid to rescue overgrowth of the *Δwhi2* strain in low leucine condition has opened questions about the role of *WHI2* in cell growth control. However, little is known roles of glutamic acid in cellular growth or function. One possible mechanism through which glutamic acid regulates cellular function is as a signaling molecule in its ionized form, glutamate. In neurons, glutamate binds to the excitatory receptors, alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-methyl-D-aspartate (NMDA), and results in in the opening of ion channels, specifically for K+, Ca+, and Na+ [58]. Glutamate signaling to these receptors can lead to increased depolarization and neuronal activity, which may play a role in epileptic seizures [58]. Additionally, GAD (glutamate decarboxylase) glutamate

can also be metabolized to GABA (gamma-amino-butyrate), the predominant inhibitory neurotransmitter commonly associated with epilepsy [59]. Mutations in the KCTD proteins, the human homologue of yeast Whi2, have been shown to cause myoclonic epilepsy, this link between glutamic acid and *WHI2* via neuronal signaling is particularly interesting [60], [61]. While there have been GABA transporters found in *S. cerevisiae*, they appear to play different roles than mammalian GABA receptors [59]. In budding yeast, GABA has been shown to regulate nitrogen catabolism and may protect yeast against oxidative stress [59]. However, a role for glutamic acid acting as a cell-to-cell signaling molecule in yeast remains an open possibility.

Another possibility is that *WHI2* plays a role in the conversion of glutamic acid to α-ketoglutarate [54, 55]. This process occurs through oxidative deamination of glutamate, the ionic form of glutamic acid, by GDH (glutamate dehydrogenase) [63]. Aside from being a basic building block for amino acid synthesis, α-ketoglutarate is also an important substrate of oxidative production of ATP [24, 62]. With a lack of α-ketoglutarate, the cell cannot properly complete the citric acid cycle to produce ATP, which may stall cellular growth. This is consistent with findings showing that mutations in *whi2* have resulted in slightly dysfunctional mitochondria due to improper regulation of mitophagy [27]. Dysfunctional mitochondria would result in impaired oxidative respiration, which could also be the result of the inability to properly synthesize α-ketoglutarate and undergo the citric acid cycle. While this may be a possibility, the inability of glutamine to rescue the overgrowth phenotype appears to indicate otherwise. One of the primary sources of intracellular glutamate is from processing glutamine by PDG (phosphate-dependent glutaminase) [63]. The inability of glutamine to rescue the phenotype despite being a

precursor for glutamate may indicate the necessity of *WHI2* in processing glutamine into glutamic acid, or that the effects of glutamic acid are independent of glutamine and the α-ketoglutarate synthetic pathway. Currently, it is not clear how glutamic acid is regulating growth, but elucidating the relationship between glutamic acid and *WHI2* could provide important information into the mechanisms behind how amino acids and *WHI2* regulate growth. However, my findings suggest novel roles for glutamic acid in cellular growth and function.

One difficulty when exploring which amino acids play a role in regulating growth was the requirement of maintaining the same total concentration of all amino acids, while varying the concentration of a single amino acid and the relative concentrations between the amino acids could all potentially play a role. As it is impossible to keep the other two conditions constant when varying a single amino acid, some of the effects on growth may not be entirely due to an addition of the amino acid. For example, increasing leucine to 50 mg/L in SCD_{ME} (1200 mg/L total amino acid concentration), also changes the total amino acid concentration to 1220 mg/L, as well as the ratio of leucine to the other amino acids in the recipe (e.g. 30 mg/L Leu to 20 mg/L Met in SCD_{ME} while 50 mg/L Leu to 20 mg/L Met in SCD_{ME}[Leu(50)]). The data presented has shown that, separately, total amino acid concentration or concentration of an individual amino does not appear to have an effect on the overgrowth observed in the *Δwhi2* and ESCRT knockout strains. However, it would be impossible to test the effect of only changing one amino acid without it affecting the total amino acid concentration or its ratio with other amino acid, making a definitive conclusion difficult.

Another issue that these experiments were not fully able to address is the signaling effect of amino acids on growth versus the need for amino acids as a nitrogen nutrient source. Especially when using plates, it is also hard to differentiate between slow growth and death on the plate. While microscopy was used to determine small colony formation was present at all dilutions (data not shown), it does not rule out the possibility that amino acids are affecting a death pathway rather than, or in combination with, a growth pathway.

The dichotomy between the short-term growth and long-term growth of the WT and Δ*whi2* strains touches on the issue mentioned above. Although the Δ*whi2* strains clearly grow better than the WT strains on SCD_{ME} and SCD_{MEDD}[Glu(100)] plates, the OD_{600} values (data not shown) elucidate poorer growth of the Δ*whi2* strains in the liquid culture for these media. This phenomenon could be due to the rapid influx of nutrients when switching media, even to fresh nutrient-limited media, which drives short-term growth. Another potential explanation could be that there are different mechanisms that regulate long-term growth. However, one confounding issue is the possibility of breakthrough colonies forming in liquid culture. Breakthrough colonies can be distinguished on agar plates as colonies that have distinctly better growth than the surrounding background, possibly arising due to mutations derived from the selective pressure of nutrient stress. While it is easy to visualize on the plates, it would be very difficult to tell if the growth in liquid culture was due to a breakthrough colony representing a small, unrepresentative fraction of the total cells rather than the majority.

Future Directions

This work provides important groundwork for further projects to not only explore nutrient signaling and sensing, but also the roles of *WHI2* may play in *S. cerevisiae*. Most importantly, it lays a basis for considering the combination of amino acids that regulate cellular growth in yeast. While it was shown that the ratio of leucine relative to a subset of amino acids is sensed in regulating growth, the composition of this subset of amino acids has yet to be identified. Moving forward, identifying the amino acids that are sensed with leucine has the largest implications in understanding amino acid regulation of cellular growth. Additionally, identifying the role that *WHI2* plays in growth regulation by amino acids could have implications in improving health, especially in situation where growth is improperly regulated. By transforming constitutively active or suppressed proteins in the TOR pathway to see their effect on growth in the Δ*whi2* strain could provide more information about the role of Whi2 in the TOR pathway. For example, if transforming a constitutively active Gtr1 protein into a *whi2* knockout strain rescues the overgrowth phenotype would imply that Whi2 is upstream of Gtr1 in the TOR pathway. In particular, a better understanding of *WHI2's* by looking at the effect on growth by adding constitutively active or inactive constructs of proteins known in the upstream TOR pathway to the Δ*whi2* strain. Similar experiments have been done by previous members in the lab, in particular a project pursued by a rotation student looking at the relation between *WHI2* and Gtr1.

Elucidating the amino acids involved in signaling TOR activation could have interesting implications in health due to the multiple roles that TOR plays in health. Aside from research that has implicated TOR dysregulation as a potential source of

tumorigenesis [6], it has also been shown to have a role in epilepsy, where human mutations in the mTORC1 inhibitors TSC1/2 promote epilepsy [55, 56]. In the realm of infectious diseases, activating mTORC1 has been shown to regulate T-helper cell differentiation [66]. If the amino acids involved in activating TORC1 could be determined, it may be possible to utilize different amino acid combinations to encourage Th1 or Th2 differentiation. This could be potentially useful to induce appropriate immune responses against certain pathogens, especially in cases of chronic infections. In some cases, T-cell exhaustion can lead to a shift from the Th1 driven pathogen clearance to a more Th2 regulated tolerance; a shift back towards a Th1 response could help drive clearance of these infections [66].

Learning more about Whi2 would also provide additional insight into the TOR pathway as well. In particular, having shown that Whi2 appears to play a role in glutamic acid metabolism, can narrow the pathways that Whi2 is in. Being able to target Whi2's role in the TORC1 pathway could be have similar implications on health and disease as mentioned above. In addition to understanding Whi2 in the context of TOR, understanding the role of Whi2 in general would provide information into the functions of it's human analogs, the KCTD proteins. As mutations in one of the KCTD proteins, KCTD7, results in progressive myoclonic epilepsy (EPM3) in humans, understanding Whi2 may provide a better understanding of how mutations in KCTD7 result in epilepsy [58, 59]. Additionally, if TOR plays a role in development of epilepsy through KCTD7, understanding the interactions between amino acids, TOR and Whi2 may provide a therapy in which certain amino acids can mitigate the severity of the disease.

Understanding the role of Whi2 in amino acid sensing and TOR activation could have wide implications in health. Aside from the experiments outlined above, more work would be needed to gain the necessary knowledge for an impact on health to be made through this work. Regardless, with the limited knowledge surrounding both amino acid sensing and the cellular function of Whi2 the results provide a better understanding and background through which these aspects could be studied.

References

[1] A. Efeyan, W. C. Comb, and D. M. Sabatini, "Nutrient-sensing mechanisms and pathways," *Nature*, vol. 13, 2015.

[2] L. Bar-Peled and D. M. Sabatini, "Regulation of mTORC1 by amino acids," *Trends Cell Biol.*, vol. 24, no. 7, pp. 400–406, Jul. 2014.

[3] S. G. Kim, G. R. Buel, and J. Blenis, "Nutrient regulation of the mTOR Complex 1 signaling pathway," *Mol. Cells*, pp. 1–11, 2013.

[4] K. Inoki and K. L. Guan, "Complexity of the TOR signaling network," *Trends Cell Biol.*, vol. 16, no. 4, pp. 206–212, 2006.

[5] M. Laplante and D. M. Sabatini, "mTOR signaling," *Cold Spring Harb. Perspect. Biol.*, vol. 4, pp. 2009–2011, 2012.

[6] M. Laplante and D. M. Sabatini, "mTOR signaling in growth control and disease," *Cell*, vol. 149, no. 2, pp. 274–293, 2013.

[7] E. Cuyàs, B. Corominas-faja, J. Joven, and J. A. Menendez, *Cell Cycle Control*, vol. 1170. 1995.

[8] N. Hay and N. Sonenberg, "Upstream and downstream of mTOR," *Genes and Development*, vol. 18, no. 16. pp. 1926–1945, 2004.

[9] P. J. Aspuria, T. Sato, and F. Tamanoi, "The TSC/Rheb/TOR signaling pathway in fission yeast and mammalian cells: Temperature sensitive and constitutive active mutants of TOR," *Cell Cycle*, vol. 6, no. February 2015, pp. 1692–1695, 2007.

[10] A. Toker and S. Marmiroli, "Signaling specificity in the Akt pathway in biology and disease," *Adv. Biol. Regul.*, vol. 55, pp. 28–38, 2014.

[11] P. S. Ward and C. B. Thompson, "Signaling in control of cell growth and metabolism.," *Cold Spring Harb. Perspect. Biol.*, vol. 4, no. 7, p. a006783, Jul. 2012.

[12] D. J. Klionsky and E. L. Eskelinen, "The vacuole versus the lysosome: When size matters," *Autophagy*, vol. 10, no. 2, pp. 185–187, 2014.

[13] A. Efeyan, R. Zoncu, and D. M. Sabatini, "Amino acids and mTORC1: From lysosomes to disease," *Trends Mol. Med.*, vol. 18, no. 9, pp. 524–533, 2012.

[14] J. Brown, K. Inoue, Y. Kimata, M. Lamers, B. Luisi, N. Lukashchuk, R. Nishi, C. Sage, and C. Schmidt, "Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1," vol. 347, no. 6218, 2015.

[15] P. Matile and A. Wiemken, “The vacuole as the lysosome of the yeast cell,” *Arch. f??r Mikrobiol.*, vol. 56, no. 2, pp. 148–155, 1967.

[16] a Wiemken and M. Dürr, “Characterization of amino acid pools in the vacuolar compartment of Saccharomyces cerevisiae.,” *Arch. Microbiol.*, vol. 101, no. 1, pp. 45–57, 1974.

[17] M. Shimazu, T. Sekito, K. Akiyama, Y. Ohsumi, and Y. Kakinuma, “A family of basic amino acid transporters of the vacuolar membrane from Saccharomyces cerevisiae,” *J. Biol. Chem.*, vol. 280, no. 6, pp. 4851–4857, 2005.

[18] C. Betz and M. N. Hall, “Where is mTOR and what is it doing there?,” *J. Cell Biol.*, vol. 203, no. 4, pp. 563–574, 2013.

[19] T. Sekiguchi, Y. Kamada, N. Furuno, M. Funakoshi, and H. Kobayashi, “Amino acid residues required for Gtr1p-Gtr2p complex formation and its interactions with the Ego1p-Ego3p complex and TORC1 components in yeast,” *Genes to Cells*, vol. 19, pp. 449–463, 2014.

[20] H. Kim, A. Kim, and K. W. Cunningham, “Vacuolar H+-ATPase (V-ATPase) promotes vacuolar membrane permeabilization and nonapoptotic death in stressed yeast,” *J. Biol. Chem.*, vol. 287, no. 23, pp. 19029–19039, 2012.

[21] L. Bar-Peled, L. Chantranupong, A. D. Cherniack, W. W. Chen, K. a Ottina, B. C. Grabiner, E. D. Spear, S. L. Carter, M. Meyerson, and D. M. Sabatini, “A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1.,” *Science*, vol. 340, no. 6136, pp. 1100–6, May 2013.

[22] N. Panchaud, M.-P. Péli-Gulli, and C. De Virgilio, “Amino acid deprivation inhibits TORC1 through a GTPase-activating protein complex for the Rag family GTPase Gtr1.,” *Sci. Signal.*, vol. 6, no. 277, p. ra42, May 2013.

[23] J. E. Hughes Hallett, X. Luo, and A. P. Capaldi, “State Transitions in the TORC1 Signaling Pathway and Information Processing in Saccharomyces cerevisiae.,” *Genetics*, vol. 198, no. October, pp. 773–786, 2014.

[24] S. Zaman, S. I. Lippman, X. Zhao, and J. R. Broach, “How Saccharomyces responds to nutrients.,” *Annu. Rev. Genet.*, vol. 42, pp. 27–81, 2008.

[25] Y. Kamada, K. Yoshino, C. Kondo, T. Kawamata, N. Oshiro, K. Yonezawa, and Y. Ohsumi, “Tor directly controls the Atg1 kinase complex to regulate autophagy.,” *Mol. Cell. Biol.*, vol. 30, no. 4, pp. 1049–1058, 2010.

[26] T. Yorimitsu, C. He, K. Wang, and D. J. Klionsky, “Tap42-associated protein phosphatase type 2A negatively regulates induction of autophagy,” *Autophagy*, vol. 5, no. March 2015, pp. 616–624, 2009.

[27] N. Mendl, A. Occhipinti, M. Müller, P. Wild, I. Dikic, and A. S. Reichert, "Mitophagy in yeast is independent of mitochondrial fission and requires the stress response gene WHI2.," *J. Cell Sci.*, vol. 124, pp. 1339–1350, 2011.

[28] D. J. Klionsky, "The molecular machinery of autophagy: unanswered questions.," *J. Cell Sci.*, vol. 118, no. Pt 1, pp. 7–18, 2005.

[29] D. J. Saul and P. E. Sudbery, "Molecular cloning of WHI2, a gene involved in the regulation of cell proliferation in Saccharomyces cerevisiae.," *J. Gen. Microbiol.*, vol. 131, pp. 1797–1806, 1985.

[30] H. a Mountain and P. E. Sudbery, "Regulation of the Saccharomyces cerevisiae WHI2 gene.," *J. Gen. Microbiol.*, vol. 136, pp. 727–732, 1990.

[31] H. a Mountain and P. E. Sudbery, "The relationship of growth rate and catabolite repression with WHI2 expression and cell size in Saccharomyces cerevisiae.," *J. Gen. Microbiol.*, vol. 136, pp. 733–737, 1990.

[32] P. a. Radcliffe, K. M. Binley, J. Trevethick, M. Hall, and P. E. Sudbery, "Filamentous growth of the budding yeast Saccharomyces cerevisiae induced by overexpression of the WHI2 gene," *Microbiology*, vol. 143, no. 1 997, pp. 1867–1876, 1997.

[33] W.-C. Cheng, X. Teng, H. K. Park, C. M. Tucker, M. J. Dunham, and J. M. Hardwick, "Fis1 deficiency selects for compensatory mutations responsible for cell death and growth control defects.," *Cell Death Differ.*, vol. 15, no. 12, pp. 1838–1846, 2008.

[34] X. Teng, M. Dayhoff-Brannigan, W. C. Cheng, C. Gilbert, C. Sing, N. Diny, S. Wheelan, M. Dunham, J. Boeke, F. Pineda, and J. M. Hardwick, "Genome-wide consequences of deleting any single gene," *Mol. Cell*, vol. 52, no. 4, pp. 485–494, 2013.

[35] A. Basak and C. C. Query, "A pseudouridine residue in the spliceosome core is part of the filamentous growth program in yeast.," *Cell Rep.*, vol. 8, no. 4, pp. 966–73, Aug. 2014.

[36] A. Sadeh, N. Movshovich, M. Volokh, L. Gheber, and A. Aharoni, "Fine-tuning of the Msn2/4-mediated yeast stress responses as revealed by systematic deletion of Msn2/4 partners.," *Mol. Biol. Cell*, vol. 22, pp. 3127–3138, 2011.

[37] P. Radcliffe, J. Trevethick, M. Tyers, and P. Sudbery, "Deregulation of CLN1 and CLN2 in the Saccharomyces cerevisiae whi2 mutant," *Yeast*, vol. 13, pp. 707–715, 1997.

[38] T. Shintani and D. J. Klionsky, "Autophagy in health and disease: a double-edged sword.," *Science*, vol. 306, no. 5698, pp. 990–995, 2004.

[39] Y. Feng, D. He, Z. Yao, and D. J. Klionsky, "The machinery of macroautophagy.," *Cell Res.*, vol. 24, no. 1, pp. 24–41, 2014.

[40] J. B. Kunzt, H. Schwarz, and A. Mayer, "Determination of Four Sequential Stages during Microautophagy in Vitro," *J. Biol. Chem.*, vol. 279, no. 11, pp. 9987–9996, 2004.

[41] M. Müller and A. S. Reichert, “Mitophagy, mitochondrial dynamics and the general stress response in yeast,” *Biochem. Soc. Trans.*, vol. 39, pp. 1514–1519, 2011.

[42] Y. Fannjiang, W. C. Cheng, S. J. Lee, B. Qi, J. Pevsner, J. M. McCaffery, R. B. Hill, G. Basanez, J. M. Hardwick, and G. Basañez, “Mitochondrial fission proteins regulate programmed cell death in yeast,” *Genes Dev*, vol. 18, no. 22, pp. 2785–2797, 2004.

[43] T. Kanki, K. Furukawa, and S. Yamashita, “Mitophagy in yeast: Molecular mechanisms and physiological role,” *Biochim. Biophys. Acta - Mol. Cell Res.*, 2015.

[44] K. Mao, K. Wang, X. Liu, and D. Klionsky, “The scaffold protein Atg11 recruits fission machinery to drive selective mitochondria degradation by autophagy,” *Dev. Cell*, vol. 26, no. 1, pp. 9–18, 2013.

[45] W. M. Henne, N. J. Buchkovich, and S. D. Emr, “The ESCRT Pathway,” *Dev. Cell*, vol. 21, no. 1, pp. 77–91, Jul. 2011.

[46] C. Sing, “ROLE FOR THE ESCRT COMPLEX IN THE RELATIONSHIP BETWEEN NUTRIENT SIGNALING AND AUTOPHAGY IN SACCHARYOMYCES CEREVISIAE,” 2014.

[47] V. R. Youhg, “Alfred E . Harper Symposium on Emerging Aspects of Amino Acid Metabolism Adult Amino Acid Requirements : The Case for a Major Revision in Current Recommendations1,” 1994.

[48] J. D. Kopple and M. E. Swendseid, “Evidence that histidine is an essential amino acid in normal and chronically uremic man,” *J. Clin. Invest.*, vol. 55, no. 5, pp. 881–891, 1975.

[49] J. T. Pronk, “Auxotrophic Yeast Strains in Fundamental and Applied Research MINIREVIEWS Auxotrophic Yeast Strains in Fundamental and Applied Research,” *Appl. Environ. Microbiol.*, vol. 68, no. 5, pp. 2095–2100, 2002.

[50] F. Sherman, “Getting started with yeast,” *Methods Enzymol.*, vol. 350, pp. 3–41, 2002.

[51] Dayhoff-B and M. Rannigan, “Identifying novel genes involved in amino acid sensing,” 2014.

[52] N. Alic, V. J. Higgins, and I. W. Dawes, “Identification of a Saccharomyces cerevisiae gene that is required for G1 arrest in response to the lipid oxidation product linoleic acid hydroperoxide.,” *Mol. Biol. Cell*, vol. 12, no. 6, pp. 1801–1810, 2001.

[53] C. Ruckenstuhl, C. Netzberger, I. Entfellner, D. Carmona-Gutierrez, T. Kickenweiz, S. Stekovic, C. Gleixner, C. Schmid, L. Klug, A. G. Sorgo, T. Eisenberg, S. Büttner, G. Mariño, R. Koziel, P. Jansen-Dürr, K.-U. Fröhlich, G. Kroemer, and F. Madeo, “Lifespan extension by methionine restriction requires autophagy-dependent vacuolar acidification.,” *PLoS Genet.*, vol. 10, no. 5, p. e1004347, May 2014.

[54] J. E. Johnson and F. B. Johnson, “Methionine restriction activates the retrograde response and confers both stress tolerance and lifespan extension to yeast, mouse and human cells.,” *PLoS One*, vol. 9, no. 5, p. e97729, Jan. 2014.

[55] D. Stracka, S. Jozefczuk, F. Rudroff, U. Sauer, and M. N. Hall, “Nitrogen source activates TOR (target of rapamycin) complex 1 via glutamine and independently of Gtr/Rag proteins.,” *J. Biol. Chem.*, vol. 289, no. 36, pp. 25010–20, Sep. 2014.

[56] Z. Wu, L. Song, S. Q. Liu, and D. Huang, “Independent and additive effects of glutamic acid and methionine on yeast longevity.,” *PLoS One*, vol. 8, no. 11, p. e79319, Jan. 2013.

[57] Y. J. Lee, K. J. Kim, H. Y. Kang, H. R. Kim, and P. J. Maeng, “Involvement of GDH3-encoded NADP+-dependent glutamate dehydrogenase in yeast cell resistance to stress-induced apoptosis in stationary phase cells,” *J. Biol. Chem.*, vol. 287, no. 53, pp. 44221–44233, 2012.

[58] M. DiNuzzo, S. Mangia, B. Maraviglia, and F. Giove, “Physiological bases of the K+ and the glutamate/GABA hypotheses of epilepsy,” *Epilepsy Res.*, vol. 108, no. 6, pp. 995–1012, 2014.

[59] Ľ. Nižňanský, S. Kryštofová, P. Vargovič, M. Kaliňák, M. Šimkovič, and Ľ. Varečka, “Glutamic acid decarboxylase gene disruption reveals signalling pathway(s) governing complex morphogenic and metabolic events in Trichoderma atroviride,” *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.*, vol. 104, no. 5, pp. 793–807, 2013.

[60] M. Kousi, V. Anttila, a. Schulz, S. Calafato, E. Jakkula, E. Riesch, L. Myllykangas, H. Kalimo, M. Topcu, S. Gokben, F. Alehan, J. R. Lemke, M. Alber, a. Palotie, O. Kopra, and a.-E. Lehesjoki, “Novel mutations consolidate KCTD7 as a progressive myoclonus epilepsy gene,” *J. Med. Genet.*, vol. 49, no. 6, pp. 391–399, 2012.

[61] S. M. K. Farhan, L. M. Murphy, J. F. Robinson, J. Wang, V. M. Siu, C. A. Rupar, A. N. Prasad, and R. a. Hegele, “Linkage analysis and exome sequencing identify a novel mutation in KCTD7 in patients with progressive myoclonus epilepsy with ataxia,” *Epilepsia*, pp. 106–111, 2014.

[62] P. Niu, X. Dong, Y. Wang, and L. Liu, “Enzymatic production of a-ketoglutaric acid from l-glutamic acid via l-glutamate oxidase,” *J. Biotechnol.*, vol. 179, no. 1, pp. 56–62, 2014.

[63] J. R. Treberg, S. Banh, U. Pandey, and D. Weihrauch, “Intertissue differences for the role of glutamate dehydrogenase in metabolism,” *Neurochem. Res.*, vol. 39, no. 3, pp. 516–526, 2014.

[64] P. Curatolo, “Mechanistic Target of Rapamycin (mTOR) in Tuberous Sclerosis Complex-Associated Epilepsy,” *Pediatr. Neurol.*, vol. 52, no. 3, pp. 281–289, 2014.

[65] L. H. Zeng, N. R. Rensing, B. Zhang, D. H. Gutmann, M. J. Gambello, and M. Wong, “Tsc2 gene inactivation causes a more severe epilepsy phenotype than Tsc1 inactivation in a mouse model of Tuberous Sclerosis Complex,” *Hum. Mol. Genet.*, vol. 20, no. 3, pp. 445–454, 2011.

[66] H. Liu, H. Yang, X. Chen, Y. Lu, Z. Zhang, J. Wang, M. Zhang, L. Xue, F. Xue, and G. Liu, “Cellular metabolism modulation in T lymphocyte immunity.,” *Immunology*, 2014.

Appendix 1: Modified SCD recipes and experimental replicate information

	SCD_{CSH}	SCD_{ME}	SCD_{Dawes}	SCD_{MEDD}	SCD_{Awe}	SCD_{CSH}-Ura	SCD_{CSH} [Leu(30)]	SCD_{ME} [Leu(300)]	SCD_{ME}[Up]	SCD_{CSH} [Leu(40)]	SCD_{CSH} [Leu(50)]	SCD_{CSH} [Leu(60)]
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Adenine	18.35	20.00	10.00	10.00	0.00	18.35	18.35	20.00	33.33	18.35	18.35	18.35
L-alanine	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-arginine	73.40	20.00	50.00	20.00	0.00	73.40	73.40	20.00	33.33	73.40	73.40	73.40
L-asparagine	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-aspartic acid	73.40	100.00	80.00	80.00	0.00	73.40	73.40	100.00	166.67	73.40	73.40	73.40
L-cysteine	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-glutamine	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-glutamic acid	73.40	100.00	0.00	0.00	0.00	73.40	73.40	100.00	166.67	73.40	73.40	73.40
L-glycine	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-histidine	73.40	20.00	20.00	20.00	20.00	73.40	73.40	20.00	33.33	73.40	73.40	73.40
Inositol	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-isoleucine	73.40	30.00	50.00	30.00	0.00	73.40	73.40	30.00	50.00	73.40	73.40	73.40
L-leucine	367.00	30.00	100.00	30.00	30.00	367.00	30.00	300.00	50.00	40.00	50.00	60.00
L-lysine	73.40	30.00	50.00	30.00	20.00	73.40	73.40	30.00	50.00	73.40	73.40	73.40
L-methionine	73.40	20.00	20.00	20.00	20.00	73.40	73.40	20.00	33.33	73.40	73.40	73.40
PABA	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-phenylalanine	73.40	50.00	50.00	50.00	0.00	73.40	73.40	50.00	83.33	73.40	73.40	73.40
L-proline	73.40	0.00	0.00	0.00	0.00	73.40	73.40	0.00	0.00	73.40	73.40	73.40
L-serine	73.40	400.00	0.00	0.00	0.00	73.40	73.40	400.00	666.67	73.40	73.40	73.40
L-threonine	73.40	200.00	100.00	100.00	0.00	73.40	73.40	200.00	333.33	73.40	73.40	73.40
L-tryptophan	73.40	20.00	100.00	20.00	0.00	73.40	73.40	20.00	33.33	73.40	73.40	73.40
L-tyrosine	73.40	30.00	50.00	30.00	0.00	73.40	73.40	30.00	50.00	73.40	73.40	73.40
Uracil	73.40	20.00	20.00	20.00	20.00	0.00	73.40	20.00	33.33	73.40	73.40	73.40
L-valine	73.40	150.00	140.00	140.00	0.00	73.40	73.40	150.00	250.00	73.40	73.40	73.40
Total	1761.60	1200.00	810.00	570.00	90.00	1761.60	1424.60	1470.00	2000.00	1434.60	1444.60	1454.60
Number of independent experiments	>5	>5	5	>5	>5	n/a	>5	3	>5	5	5	5
Number of different plate batches made	>3	>3	1	>3	3	n/a	2	2	>3	2	2	2

	SCD_{ME} [Leu(50)]	SCD_{ME} [Leu(75)]	SCD_{ME} [Leu(100)]	SCD_{Aux} [20% Leu]	SCD_{Aux} [9.09% Leu]	SCD_{Aux} [2.5% Leu]	SCD_{Aux} [Met(10)]	SCD_{Aux} [Met(100)]	SCD_{Aux} [Arg(20)]	SCD_{Aux} [Asp(100)]	SCD_{Aux} [Ile(30)]	SCD_{Aux} [Phe(50)]
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Adenine	20.00	20.00	20.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-alanine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-arginine	20.00	20.00	20.00	0.00	0.00	0.00	0.00	0.00	20.00	0.00	0.00	0.00
L-asparagine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	80.00	0.00	0.00
L-aspartic acid	100.00	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-cysteine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glutamine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glutamic acid	100.00	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glycine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-histidine	20.00	20.00	20.00	40.00	100.00	390.00	10.00	100.00	20.00	20.00	20.00	20.00
Inositol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-isoleucine	30.00	30.00	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	30.00	0.00
L-leucine	50.00	75.00	100.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
L-lysine	30.00	30.00	30.00	40.00	100.00	390.00	20.00	20.00	20.00	20.00	20.00	20.00
L-methionine	20.00	20.00	20.00	40.00	100.00	390.00	20.00	20.00	20.00	20.00	20.00	20.00
PABA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-phenylalanine	50.00	50.00	50.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	50.00
L-proline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-serine	400.00	400.00	400.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-threonine	200.00	200.00	200.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-tryptophan	20.00	20.00	20.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-tyrosine	30.00	30.00	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Uracil	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
L-valine	150.00	150.00	150.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	1220.00	1245.00	1270.00	150.00	330.00	1200.00	80.00	170.00	110.00	170.00	120.00	140.00
Number of independent experiments	>5	4	4	3	3	3	3	3	3	3	3	3
Number of different plate batches made	2	2	2	1	1	1	1	1	2	2	2	2

	SCD_{Aux} [Thr(100)]	SCD_{Aux} [Trp(20)]	SCD_{Aux} [Tyr(30)]	SCD_{Aux} [Val(30)]	SCD_{Aux} [RDTV]	SCD_{Aux} [IFV]	SCD_{Aux} [IFWY]	SCD_{Aux} [TYV]	SCD_{Aux} [RDWY]	SCD_{Aux} [RDIFT]	SCD_{Aux} [TWY]	SCD_{MEDD} [DESTV]
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Adenine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.00
L-alanine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-arginine	0.00	0.00	0.00	0.00	20.00	0.00	0.00	0.00	20.00	20.00	0.00	20.00
L-asparagine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-aspartic acid	0.00	0.00	0.00	0.00	80.00	0.00	0.00	0.00	80.00	80.00	0.00	100.00
L-cysteine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glutamine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glutamic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00
L-glycine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-histidine	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Inositol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-isoleucine	0.00	0.00	0.00	0.00	0.00	30.00	30.00	0.00	0.00	30.00	0.00	30.00
L-leucine	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
L-lysine	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	30.00
L-methionine	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
PABA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-phenylalanine	0.00	0.00	0.00	0.00	0.00	50.00	50.00	0.00	0.00	50.00	0.00	50.00
L-proline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-serine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	400.00
L-threonine	100.00	0.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	100.00	100.00	200.00
L-tryptophan	0.00	20.00	0.00	0.00	0.00	0.00	20.00	0.00	20.00	0.00	20.00	20.00
L-tyrosine	0.00	0.00	30.00	0.00	0.00	0.00	30.00	30.00	30.00	0.00	30.00	30.00
Uracil	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
L-valine	0.00	0.00	0.00	140.00	140.00	140.00	0.00	140.00	0.00	0.00	0.00	150.00
Total	190.00	110.00	120.00	230.00	430.00	310.00	220.00	360.00	240.00	370.00	240.00	1200.00
Number of independent experiments	3	3	2	3	2	2	2	2	2	2	2	3
Number of different plate batches made	2	2	1	2	1	1	1	1	1	1	1	1

	SCD_{MEDD} [EST]	SCD_{MEDD} [Glu(100)]	SCD_{MEDD} [Ala(100)]	SCD_{MEDD} [Asn(100)]	SCD_{MEDD} [Cys (100)]	SCD_{MEDD} [Gln(100)]	SCD_{MEDD} [Gly(100)]	SCD_{MEDD} [Pro(100)]	SCD_{MEDD} [Ser(400)]	SCD_{MEDD} [Thr(200)]	SCD_{MEDD} [670 tot]	SCD_{MEDD} [Asp(180)]
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Adenine	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	11.75	10.00
L-alanine	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-arginine	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	23.51	20.00
L-asparagine	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-aspartic acid	80.00	80.00	80.00	80.00	80.00	80.00	80.00	80.00	80.00	80.00	94.04	180.00
L-cysteine	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glutamine	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glutamic acid	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-glycine	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00
L-histidine	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	23.51	20.00
Inositol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-isoleucine	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	35.26	30.00
L-leucine	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	35.26	30.00
L-lysine	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	35.26	30.00
L-methionine	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	23.51	20.00
PABA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L-phenylalanine	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	58.77	50.00
L-proline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00
L-serine	400.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	400.00	0.00	0.00	0.00
L-threonine	200.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	200.00	117.54	100.00
L-tryptophan	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	23.51	20.00
L-tyrosine	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	35.26	30.00
Uracil	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	23.51	20.00
L-valine	140.00	140.00	140.00	140.00	140.00	140.00	140.00	140.00	140.00	140.00	164.56	140.00
Total	1170.00	670.00	670.00	670.00	670.00	670.00	670.00	670.00	970.00	670.00	670.00	670.00
Number of independent experiments	4	>5	3	3	1	3	3	3	3	3	3	3
Number of different plate batches made	1	3	2	2	1	2	2	2	2	2	2	2

	SCD_{MEDD} [Leu(100)]
	mg/L
Adenine	10.00
L-alanine	0.00
L-arginine	20.00
L-asparagine	0.00
L-aspartic acid	80.00
L-cysteine	0.00
L-glutamine	0.00
L-glutamic acid	0.00
L-glycine	0.00
L-histidine	20.00
Inositol	0.00
L-isoleucine	30.00
L-leucine	130.00
L-lysine	30.00
L-methionine	20.00
PABA	0.00
L-phenylalanine	50.00
L-proline	0.00
L-serine	0.00
L-threonine	100.00
L-tryptophan	20.00
L-tyrosine	30.00
Uracil	20.00
L-valine	140.00
Total	670.00
Number of independent experiments	3
Number of different plate batches made	2

Curriculum Vitae

E R I C Y A U

1 E. Chase Street, Apt 408 ♦ Baltimore, MD 21202 ♦ (714)335-0289 ♦ e.yau628@gmail.com

EDUCATION

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD Aug 2013-Present
Masters of Science Candidate in Molecular Microbiology and Immunology

Johns Hopkins University, Baltimore, MD September 2009-May 2013
Bachelor of Arts in Public Health Studies, Bachelor of Arts in Chemistry

RESEARCH EXPERIENCE

Johns Hopkins Bloomberg School of Public Health Graduate Research
Department of Molecular Microbiology and Immunology 01/2014 - Present
Research Advisor: Dr. Marie J Hardwick || Project Title: Effect of amino acids on TOR activation and autophagy through *whi2* and the ESCRT complex

- Tested the effects of amino acids on the growth of *Saccharomyces cerevisiae* knock out mutants by performing plating, serial dilutions, and plate imaging
- Utilized fluorescence microscopy to detect Whi2 protein localization and association with autophagy markers (e.g. LC3)
- Identified and reconfirmed subcellular co-localization of KCTD9 protein with various endosomal structures, in particular with multivesicular bodies (MVBs)
- Mentored a first year master's student in simple experiments
- Participated and presented in weekly, two-hour, lab meetings

Research Advisor: Dr. Ying Zhang || Project Title: Differences in PhoU Localization and Expression in *Escherichia coli* Lab strain W3110 and Pathogenic strain UTI89

- Measured cell forming units (CFU) to show higher bacterial persistence of pathogenic patient-isolated *E. coli* UTI89 than the non-pathogenic lab strain W3110
- Cloned a red fluorescent Protein (RFP) tagged UTI89 *E. coli* phoU used in fluorescence microscopy to detect localization of the protein
- Evaluated PhoU protein expression through immunoblotting, fluorescence microplate assays, and fluorescence microscopy
- Presented the results of the project in a 30 minute presentation in the department-wide forum

Johns Hopkins Bloomberg School of Public Health Undergraduate Research Assistant
Department of Molecular Microbiology and Immunology 01/2012-06/2013
Research Advisor: Dr. Marie J Hardwick || Project Title: Metal binding affinity of patient-mutated KCTD7 and wild-type KCTD7, characterization of KCTD5

- Purified glutathione-tagged KCTD7 protein from transformed BL21 competent cells using a Glutathione S Transferase (GST) bead pull-down and cleavage
- Showed that the patient-mutant KCTD7 showed a lower capability to bind zinc and copper with a metal binding assay
- Tested using an immunofluorescence assay to show that with and without mitochondrial damage induced by Carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) KCTD5 did not show similar cellular localization patterns as KCTD7; without inducing mitochondrial depolarization, KCTD5 was seen to be diffuse throughout the cytoplasm, but could no longer be detected after CCCP treatment

Johns Hopkins Medical Institution Summer Journal Club
Cancer Research Building 06/2010-08/2010
Mentor: Dr. Tzyy-Choou Wu || HPV Vaccine Development and Immunology Journal Club

- Analyzed primary literature to obtain knowledge about the immune system development and regulation, vaccine development, and cancer biogenesis
- Developed skills in presenting primary literature and discussing HPV, vaccine development and immunology

LEADERSHIP & OUTREACH EXPERIENCES

Generation Tomorrow/STAR TRACK HIV/HCV Tester and Counselor
Center for AIDS Research; Baltimore, Maryland 09/2014- Present

- Refreshed training as an HIV tester and counselor and became also became trained as an HCV tester and counselor for the state of Maryland
- Performed HIV and HCV rapid tests to increase detection of previously untested individuals, and helped link them to care services
- Conducted outreach and awareness events by passing out pamphlets and other brochures and staffing information tables to increase awareness of HIV and HCV in the eastern Baltimore community
- Attended a semi-monthly speaker series in order to learn more about HIV and HCV topics, such as epidemiology, treatments, social stigma, and prevention, etc.

Sisters Together and Reaching (STAR) HIV/HCV Tester and Outreach
Baltimore, Maryland 09/2013- 05/ 2014

- Trained as an HIV tester and counselor for the state of Maryland
- Promoted HIV awareness through informational booths held at health fairs targeted towards the underserved African-American population in Eastern Baltimore.

Taiwanese American Students Association (TASA) Co-President
Johns Hopkins University 05/2011 - 05/ 2013

- Planned cultural outreach events on campus, including reserving venues and equipment, contacting organizations and individuals such as Youtube stars for collaborations and coordinating students to complete their shifts
- Oversaw financial, fundraising, and budgeting executions

- Established and strengthened the intercollegiate network between the Johns Hopkins Taiwanese American Students Association and other collegiate Taiwanese American Student Associations in the east coast region, in particular the mid-Atlantic region such as those from Towson University, Georgetown University, and University of Maryland College Park
- Mentored underclassmen, teaching not only club-related tasks, but also provided help and guidance with other academic and non-academic affairs

Intercollegiate Taiwanese American Students Association (ITASA) District Chair
Evanston, IL 05/2011 - 04/2012

- Coordinated networking and volunteering events for the collegiate Taiwanese Student Associations in the Maryland, Virginia, DC area
- Represented the Baltimore area Taiwanese Student Groups at the national board and East-coast regional meetings
- Helped implement and fundraise for the new outreach initiative entitled Footprints

Taiwanese American Students Association (TASA) Publicity Chair
Johns Hopkins University 05/2010 - 05/ 2011

- Created flyers and handouts, and utilized social media and e-mail notifications to publicize the various events held on and off campus
- Prepared workshops to mentor freshman TASA members, in particular with topics such as event preparation and publicity

Intercollegiate Taiwanese American Students Association (ITASA) Assistant Development Director
Evanston, IL 09/2010 - 04/2011

- Developed and executed marketing strategies to fundraise for a national 501(c)(3) non-profit organization
- Raised over $10,000 from professional partners to fund for collegiate outreach events and initiatives
- Created a new Alumni Relations department to increase alumni engagement in networking and fundraising

Patient Outreach for Joy Wellness Center Volunteer
The Shepherd's Clinic 09/2010- 12/2010

- Promoted the services of the Wellness Centers by implementing interactive sessions in the waiting room where patients could try examples of healthy foods and learn more about the Wellness Center's holistic health offerings
- Advertised and posted flyers to help promote the center's services
- Provided administrative help with coordinating the services offered at the center, as well as interacted with patients to help set up and follow up with appointments

Crossover Basketball Volunteer
Johns Hopkins University 09/2009-12/2009

- Played basketball as a way to keep children and teenagers from the East Baltimore community engaged with an after-school activity in order to connect with them through providing support and mentorship

ACHIEVEMENTS

SCIENCE

Poster Presentation

Kyle Metz, Xinchen Teng, Edda Haberlandt, Esther Leshinsky-Silver, Heather Lamb, **Eric Yau**, Lucian Soane, Constantin van Outryve d' Ydewalle, Charlotte Sumner, Isabelle Coppens, J. Marie Hardwick. Yeast Gene Whi2 Indentifies Role for Epilepsy Gene KCTD7 in Autophagy and Mitochondrial Homeostasis. Autophagy: Fundamentals to Disease (E2). Austin, TX 2014

ACADEMIC

2015-Present Delta Omega Public Health Honor Society Alpha Chapter Member
2009-Present National Society of Collegiate Scholars
Fall 2009-Spring 2010, Fall 2011 Dean's List

OTHER WORK EXPERIENCE

Johns Hopkins Student Employment Services Office Assistant
Johns Hopkins University 09/2011-05/2012

- Processed new student employments by ensuring completeness paperwork, entering data into computer database and assembling and archiving student employee files
- Answered phone calls directed to student employment office and guided walk-ins appointments with completion of tax forms or directing to appropriate personnel

Johns Hopkins Organization Development Training Database Assistant
Johns Hopkins Health Systems 09/2011-05/2012

- Developed documentation for new database organizational software
- Coded simple computer programs that departments within Johns Hopkins Health Systems used to manage employee training using the novel database software